Division of Agricultural Sciences
UNIVERSITY OF CALIFORNIA

After research it takes

THE GROWER

+

THE AGRONOMIST

+

THE CHEMIST

for an effective program

1 plant analysis

. . . a guide for sugar beet fertilization

Albert Ulrich
David Ririe
Frederick J. Hills
Alan G. George
Morton D. Morse

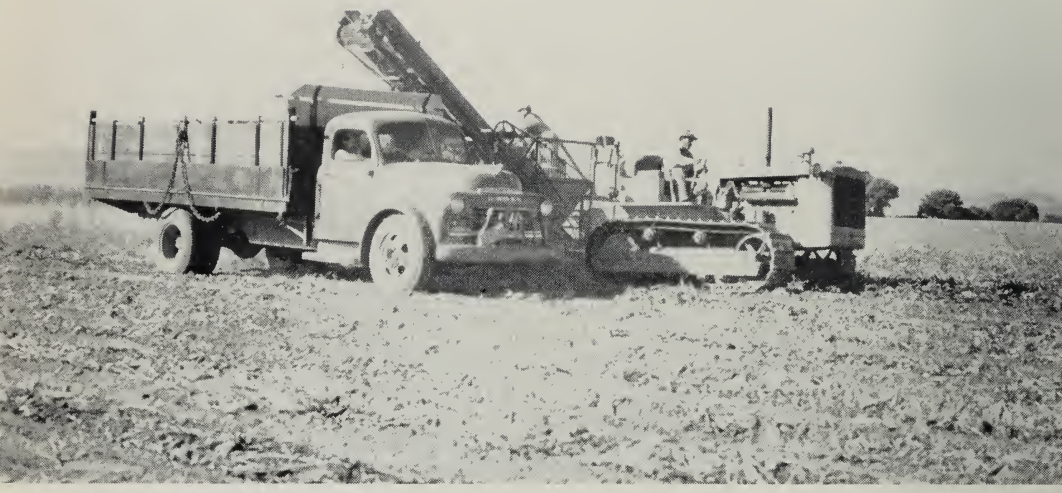
2 analytical methods

. . . for use in plant analysis

Clarence M. Johnson
Albert Ulrich

CALIFORNIA AGRICULTURAL EXPERIMENT STATION

BULLETIN 766



As successive cropping decreases the native fertility of California soils, fertilization becomes increasingly important. Efficient fertilization is possible if

1. the nutrients in short supply have been determined, and
2. these nutrients are applied in the proper amounts and at the right time and place to assure effective utilization by plants.

These requirements for efficient fertilization are difficult to determine. We are constantly looking for scientific tools to guide fertilization practices.

This bulletin, in its first part (pages 4 to 23) describes one of the newer tools of modern agriculture, plant analysis, and tells how to use it to determine the fertilization needs of sugar beets under commercial field conditions.

The second part of the bulletin (pages 25 to 77) describes the analytical methods of plant analysis for many crops as they are used in University of California laboratories.

THE AUTHORS

Albert Ulrich is Lecturer in Soils and Plant Nutrition, and Plant Physiologist in the Experiment Station; David Ririe was Assistant Professor of Agronomy and Assistant Agronomist in the Experiment Station, Davis; F. J. Hills is Agriculturist, Agricultural Extension, Department of Agronomy, Davis; Alan G. George is Assistant Agriculturist, Agricultural Extension, Tulare County; and Morton D. Morse is Associate Agriculturist, Agricultural Extension, Butte County.

Clarence M. Johnson is Chemist in Soils and Plant Nutrition, Experiment Station, Berkeley.

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I. Plant Analysis
A Guide for Sugar Beet Fertilization

CONTENTS

	Page
Growth and development of the sugar beet	4
Understanding plant growth	4
Factors increasing sugar concentration	5
Factors decreasing sugar concentration	5
Nutritional problems in California	5
Nitrogen	6
Phosphorus	6
Potassium	6
Sulfur	6
The nitrogen problem	6
Guides to fertilization	7
The field trial	7
Soil tests	7
Plant analysis	7
Development of plant analysis as a guide to fertilization	8
The theory behind plant analysis	8
Plant analysis for nitrogen	9
Efficient fertilization through plant analysis	12
Guide to nitrogen fertilization	12
Guide to harvest	14
Evaluation of fertilizer programs	15
Diagnosis of deficiency symptoms	15
Nutritional problems other than nitrogen	15
Phosphorus	15
Sulfur	17
Taking leaf samples	18
Selection of leaf	18
Routine sampling	20
Special samples	20
How often to sample	21
Preparing samples	22
Applying fertilizer	22
Form of nitrogen	22
Placement of fertilizer nitrogen	23
Phosphorus	23
Potassium	23
Foliar applications	23

I. PLANT ANALYSIS

A Guide for Sugar Beet Fertilization

ALBERT ULRICH DAVID RIRIE F. J. HILLS

ALAN G. GEORGE MORTON D. MORSE

GROWTH AND DEVELOPMENT OF THE SUGAR BEET[1]

In recent years much information has been accumulated as to how a sugar beet grows and develops. An understanding of this growth pattern helps in making decisions as to fertilization and other cultural practices.

Unless plant growth is rapid throughout most of the growing season, no sugar-beet field will produce a large crop of beet roots. Also, beets will not have a high sugar concentration unless growth, which uses sugar, is retarded several weeks before the crop is harvested. Growth retardation must not take place suddenly nor must it impair sugar formation to the extent that less sugar is being manufactured than the plant is currently using. Since rapid growth and sugar accumulation are not compatible, it is particularly important to know those conditions that contribute to the storage of sugar in the beet root.

Understanding of plant growth—a prerequisite of high sugar production

The life cycle of the sugar beet plant starts with the germination of the seed in a moist soil at a favorable temperature. As the seed germinates, it forms a fine root; this is followed by the growth of cotyledons (seed leaves) which are pushed out of the soil into the air by their hypocotyl (stem). As the cotyledons enlarge, the sugar formed in photosynthesis is used to form more fibrous roots and the first true leaves of the plant. Leaf growth and fibrous-root growth proceed at a rapid pace until the tops reach nearly their maximum size. This size depends on the genetic composition of the variety and the climate in which the plant is growing. The sucrose concentration of the roots also increases gradually until at the maximum top size, a maximum sucrose concentration characteristic for the variety and climate is attained. At this time the sugar not needed for respiration or for the formation of tops and fibrous roots is used for storage root growth. This surplus sugar, therefore, determines the amount of storage root formed by the beet plant when all other growth factors are present in abundance.

The growth and development of the sugar beet plant, as outlined, takes place in an orderly fashion as long as the climate (temperature, light, moisture) is favorable, and no nutritional deficiencies, diseases, pests, or other factors are hindering plant growth. When there is a change in climate or in the nutritional status, a new equilibrium is established within the plant. Some of these changes

[1] Submitted for publication September 25, 1957.

when they occur in the fall of the year, may induce sugar storage or "ripening," while others, especially those inducing new growth, cause a lowering of the sucrose concentration of the beet root.

Factors increasing sugar concentration

"Ripening" or "sugaring up" of the sugar beet plant may occur at any time after the true leaves are formed even though the "storage root" is no larger than the lead of a pencil.

One easy way of inducing early "ripening" consists of depriving the sugar beet plant of an adequate supply of nitrogen. Nitrogen-deficient beets, as a rule, are higher in sucrose concentration than those adequately supplied with nitrogen.

A second way to increase sugar concentration is to subject the plants to a low temperature at night for a period of several weeks. Plants, even though they are high in nitrogen, will increase in sucrose concentration under these conditions.

Still higher sucrose concentrations may be developed in sugar-beet plants by a combination of these two methods—the induction of nitrogen deficiency during periods of cool weather. This is exactly what takes place normally in the fall if excessive use of nitrogen has been avoided.

Long days are also conducive to an increase in sucrose concentration even though the plants are supplied with ample quantities of nitrogen, but the increase in sugar concentration is not nearly as great as the effect upon the size of the storage root. In a controlled experiment, sugar-beet plants in sunlight for a 14-hour day contained approximately one percentage unit more sugar than those grown in an eight-hour day. The storage roots of these plants, however, were about double the size of those for the eight-hour day. The extra sugar produced by the leaves of the plants in days of 14 hours was utilized in storage-root growth and not primarily for increasing the sucrose concentration of the root as one might have expected.

Factors decreasing sugar concentration

There are many conditions which lead to decreases in sugar concentration of the beet root. Some of these are: periods of low light intensity, relatively short days and warm nights. Even the shading of beet plants by an overgrowth of weeds and the presence of diseases and insects may lead to a lowering of the sugar concentration of the beet root. Depressing, too, are the additions of nitrogen to nitrogen-deficient plants during periods of good weather. Under these conditions nitrogen stimulates new growth and causes a rapid decrease in the sugar stored within the beet root. This is the primary action of late summer or fall rains which, in falling, wash nitrates from the soil surface into the root zone. Another unwanted source of nitrogen prior to harvest is nitrogen which often originates from the decomposition of crop residues or other organic matter of the soil. Knowledge of the effects of these phenomena as revealed by plant analysis along with information about the general growth pattern of the sugar beet plant can lead to greater sugar production under field conditions.

NUTRITIONAL PROBLEMS IN CALIFORNIA

Most sugar-beet crops in California require greater amounts of nitrogen than our soils can supply to produce maximum yields. Fertilization with nitrogen and occasionally with phosphorus, potassium and sulfur has become an exceedingly important phase of sugar beet production. Without the use of these fertilizers many of our fields could not produce enough to meet minimum costs of production. How-

ever, the problem of plant-nutrient deficiencies in California for sugar beets is relatively simple as compared to many other areas of the United States.

Nitrogen

Nitrogen fertilizers are required for good sugar beet production in nearly all of our beet growing areas. The Delta Region of the Sacramento-San Joaquin is perhaps the one exception. The peat and semi-peat soils of this area generally supply all the nitrogen needed. Frequently, sugar beets with low sugar concentrations are produced in this area. This is often associated with high nitrogen absorption just before harvest.

Phosphorus

Profitable responses to phosphorus fertilization are becoming more common in California. We can expect the need for phosphorus fertilizers to increase. At present, however, there are only two general areas of the state, the Sacramento-San Joaquin Delta and the Imperial Valley, where sugar beets on certain soils frequently require additions of phosphorus fertilizers. Recently, responses to phosphorus have been observed in Tulare and Butte counties, but the need for phosphorus fertilization in these areas is by no means general.

Potassium

Most of our soils in California are well supplied with potassium. Many field fertilizer experiments have been conducted in recent years. In only one of these, located on Ryer Island, Solano County, did sugar beets respond to potassium fertilization. In Butte County the petioles of beets in some fields have been found low in potassium. Response to potassium fertilization has not been obtained thus far, either because the beets were not low enough in potassium or the amounts absorbed from the potassium fertilizer were insufficient to correct the deficiency. The need for more study of the problem is indicated, especially now that the higher yields from nitrogen fertilizations draw more heavily on other soil nutrients, including potassium.

Sulfur

Sugar beets in the upper Sacramento Valley have been found deficient in sulfur. Sulfur deficiencies in this area usually do not occur over entire fields, but show up in localized areas—sometimes rather large ones—within a field. Experience so far indicates that the deficiency can be corrected readily by using ammonium sulfate as a nitrogen source or by applying gypsum to the soil.

With the correction of existing nutrient deficiencies and the elimination of other factors limiting sugar beet growth, higher yields can be expected which in time will exhaust the readily available supplies of nutrients of the soil. This will result in an increasing need for fertilization that must be based on specific knowledge of each field and crop if maximum production is to be obtained at the lowest possible cost.

The nitrogen problem

Nitrogen fertilization requires special attention, not only because it is the major nutrient we use in fertilizing sugar beets, but also because of the effect of nitrogen on the sucrose concentration of the beet root. It could well be that the overuse and late use of nitrogen is the major factor contributing to the decline in sucrose concentration of beets in California.

As pointed out before, sugar beets absorbing large amounts of nitrogen are lower in sugar concentration. Too much fertilizer nitrogen, therefore, penalizes the farmer in two ways:

he spends money for fertilizer his plants cannot efficiently use and

his sugar beets are worth less per ton because of a lower sugar content.

The sugar processor, too, is concerned about sugar beets harvested when they are absorbing large amounts of nitrogen.

"Harmful-nitrogen" compounds are formed in relatively larger amounts under these conditions. It is difficult to extract as much sugar from such beets as is possible when these compounds are not present at high levels. Lower sugar contents also decrease factory operating efficiency and increase hauling costs per pound of sugar extracted from the beets.

The principal problem with nitrogen fertilization is to decide how much nitrogen to apply for maximum net income. This is not easily done, even with the results of many fertilizer experiments and much experience to draw upon. The differences of soils, crops and management practices in California result in considerable variation in the nitrogen fertilizer requirement for different fields. Sugar beets grown on fields of adjoining farms may respond quite differently to nitrogen fertilization. This response may vary from none at all to the profitable use of 240 pounds or more of elemental nitrogen per acre. The same field may even respond differently in different years.

GUIDES TO FERTILIZATION

Basically, the idea of fertilizing a crop is rather simple. All that needs to be done is to add, as a fertilizer, the amount of material to make up the difference between the nutrients needed by the crop and those supplied by the soil. However, the exact quantity of fertilizer to apply is not easy to determine. The grower is dealing with a dynamic system, the outcome of which, in terms of crop production, is dependent upon many factors. The amount of growth to be made by the plant and the supply of nutrients to be delivered from the soil cannot be estimated precisely. Over the years, however, soil and plant scientists have developed and used several types of tests for estimating the nutrient requirements of crops.

The field trial

The field trial or field experiment is perhaps the oldest method developed for evaluating a new cultural practice. It still is considered decisive before adopting a new idea for enhancing crop production. Perhaps the most important objection against the field trial is that its results apply only to the spot for which results have been obtained, and only for the year (or years) for which the experiments were conducted.

There is a need for other methods of estimating the fertilizer requirements of crops—methods that might be less costly, less time consuming, and perhaps applicable on a wider scope. To meet these needs, two general types of tests have been developed—soil analysis and plant analysis.

Soil tests

Soils can be tested by chemical or biological means. Such tests estimate the concentration of a soil nutrient which is present in the soil. This concentration is used to predict the need for additional plant nutrients. The interpretation of each test is difficult as each test requires consideration of the crop, soil type, and the climate of the area. In California, relatively few soil tests have been found reliable for estimating fertilizer needs.

Plant analysis

Plants, or parts of plants, can be analyzed chemically for specific elements or nutrient compounds. The concentrations found within the plants reflect what the plant has obtained from the soil in relation to its growth up to the time of sampling the plant. By comparing these nutrient concentrations to those already established as just deficient for growth (critical nutrient concentrations) the nutrient status of the plant can be ascertained. Plants well above the critical

nutrient concentration are adequately supplied with that nutrient; plants at or below the critical nutrient concentration are deficient in that nutrient. The longer a deficiency persists, and the earlier in the growth cycle the deficiency occurs, the more likely it is that the crop will respond to the addition of the nutrient.

The importance of plant analysis to plant nutrition rests upon the fact that the nutrient concentration within the plant directly reflects the ability of the plant to grow and acquire nutrients from the soil in its own environment. This ability cannot be predicted safely from soil analysis alone, since soil analyses do not evaluate the climatic factors affecting plant growth or the kind of plant grown.

DEVELOPMENT OF PLANT ANALYSIS AS A GUIDE TO FERTILIZATION

Before plant analysis can be used as a guide to fertilization a foundation must be carefully laid. Each crop and each nutrient is a special problem unto itself but once the basis for evaluating the nutrient status of a crop has been established, the same system can be used over a wide range of soils and climatic conditions with a reasonable assurance of success. For example, in California the same critical nutrient concentration for nitrogen and for phosphorus holds for sugar beets grown in fields ranging from the Imperial Valley to the Sacramento and San Joaquin Valleys, as well as along the coast. This has been found to be true for not only one year, but for a period of more than 15 years.

The theory behind plant analysis

The central idea behind the use of plant analysis is this: an element essential for the growth of a plant must be contained in the plant, and at a concentration sufficient for plant growth. The exact concentration at which a plant becomes deficient in a nutrient will depend upon the functions which that element performs within the plant. If we knew the exact function of a nutrient, then we would know the concentration that is necessary for the plant to grow and develop. Ideally this concentration should be determined for a single cell or, at most, for a group of cells with a similar function. But the exact function of each element within the cell is not now known, thus the problem of learning what concentration is necessary for growth must be solved for the present by trial and error.

Determining the critical concentration of a nutrient by trial and error proved relatively simple, once the essential features of the determination of the critical concentration were clearly recognized. All that needs to be done is to grow plants on a soil or in a culture medium well suppled with nutrients except the one nutrient to be studied. By adding increasing doses of the deficient element to a series of field plots or to pots with the culture medium, plant growth increases until the requirements of the plants for the deficient element are fully met. The plants are harvested when there are large differences in growth due to fertilization; then they are weighed, sampled, and analyzed chemically. In practice, this means that we select leaves or parts of leaves, or stems or parts of stems for analysis. The form of the nutrient determined depends upon the element under investigation. In some instances, plant analysis deals with the total concentration of the element within the plant part; in others with the soluble or insoluble form of the nutrient; or at times with some specific compound, such as nitrate for the element nitrogen, or phosphate soluble in 2 per cent acetic acid for phosphorus. Once the critical value for a nutrient element has been established,

growers who wish to check fertilization needs in their field can collect sugar-beet leaves according to directions given on pages 18 to 22 and send them to a commercial laboratory for analysis. The following discussion on charting nutrient contents and fertilization needs may help explain the theories behind fertilization recommendations.

Plant analysis for nitrogen

A typical calibration curve for beets in a nitrogen series is depicted in Figure 1. This curve was obtained by plotting nitrate-nitrogen in the petioles (leafstalks) of recently matured leaves of sugar beet plants on the horizontal axis and the corresponding root weights on the vertical axis. The points plotted are from an experiment involving a series of nitrogen treatments in a field of beets north of Woodland, California. The petiole and beet root samples were collected on July 30. On this date the nitrate-nitrogen values for the petioles from the untreated beets are exceedingly low and do not differ greatly from each other. The beet root weights, however, do differ from each other considerably and are related to the amount of nitrogen the plants took from the soil earlier in the growing season. When nitrogen was applied to the

Critical nitrogen concentration is reached at approximately 1,000 parts per million on the dry basis. After this point (arrow) additional nitrogen application will not increase beet-root weight appreciably.

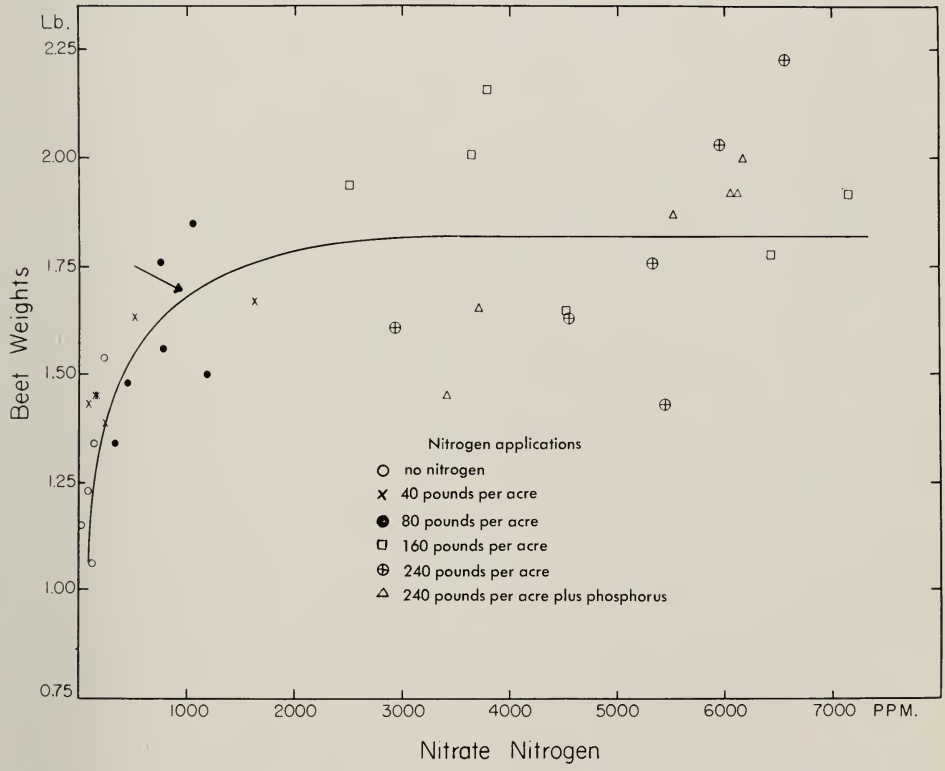

Fig. 1. Relation of beet-root weight to the nitrate-nitrogen content of petioles of recently matured leaves of sugar beet plants receiving increasing amounts of nitrogen. (From a field experiment, Woodland.)

plots at the rate of 40 lbs per acre, the nitrate concentrations of the petioles from three of the six plots did not increase significantly even though the yields increased appreciably, again indicating a shortage of nitrogen in relation to beet growth. The nitrate values and the yields increased simultaneously for three plots in the 40 lb application. For the 80 lb application, the nitrate values and yields increased simultaneously and finally for the 160 and 240 lb applications the nitrate values of the petioles increased tremendously but the beet root weights remained unaffected. From this curve it will be seen that beet root weights will increase no further after the critical point has been reached, at about 1,000 ppm. To insure the nitrate content against sinking below that point, the grower should keep nitrates up to at least 5,000 ppm or even higher than that early in the season. Such values are referred to as "safe levels" and are assumed to be indicative of an adequate supply of nutrients for the remainder of the growing season. These values will fluctuate far more than the "critical concentration," since a safe level is a prognostic value and, to have significance, assumes a constancy of climate from year to year, uniformity of soil type within a locality, and even a similarity of cultural practices from field to field.

Critical concentration in phosphate is reached at about 750 parts per million (arrow) on the dry basis.

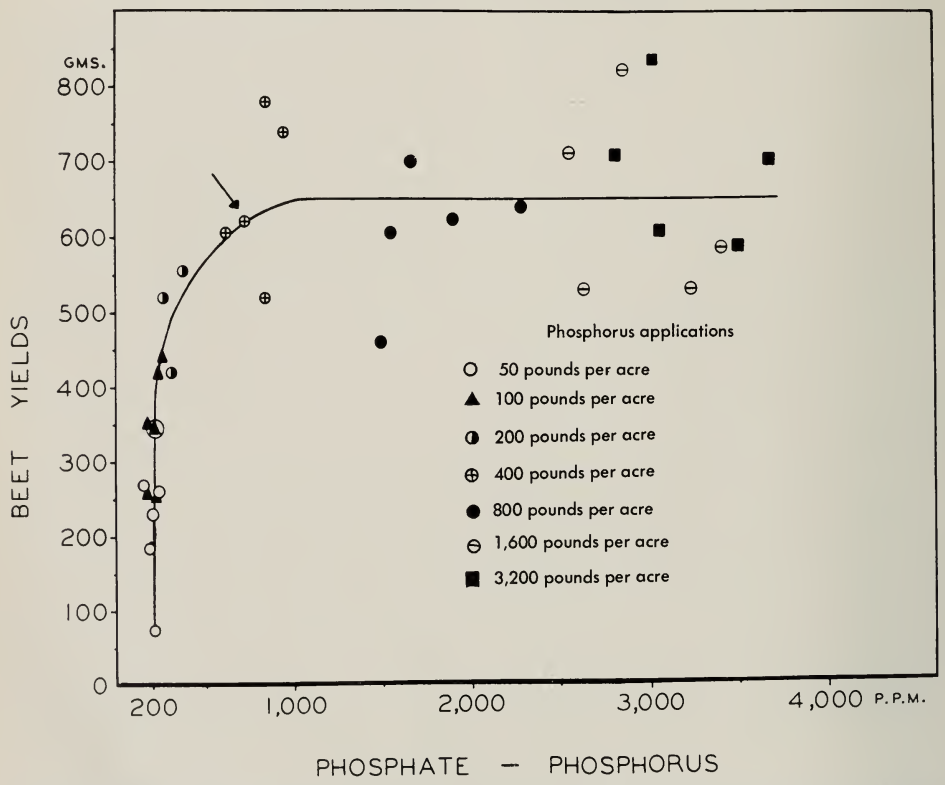

Fig. 2. Relation of beet-root weight to the concentration of phosphate-phosphorus in petioles of recently matured leaves of plants that were supplied with increasing amounts of phosphorus. (From a pot experiment, Berkeley.)

Critical potassium concentration is at about 1.0 per cent potassium on a dry basis (arrow) for either petioles or blades.

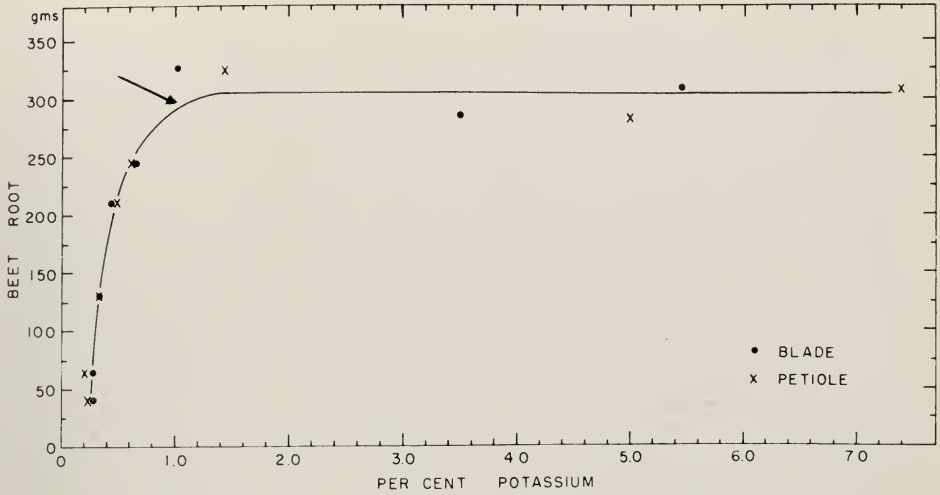

Fig. 3. Relation of beet-root weights to the potassium concentration of petioles or blades of recently matured leaves of sugar beet plants receiving increasing amounts of potassium and a constant but adequate amount of sodium. The critical potassium value of 1 per cent holds for blades regardless of sodium content, and for petioles only when the sodium concentration is greater than 1.5 per cent. Each symbol in the graph represents an average of five replications. (From a solution-culture experiment, Berkeley.)

A similar calibration curve is given for phosphorus in Figure 2. The curve was obtained by growing sugar beets in a pot experiment. Starting with a soil very low in available phosphorus, namely Aiken clay loam, and adding increasing amounts of phosphorus to the soil, the analysis of recently "matured" leaves was found to reflect the phosphorus status of the beet plant satisfactorily. The petioles of these leaves appeared to give a narrower transition zone than the leaf blades, and measurement of phosphate-phosphorus soluble in 2 per cent acetic acid was better than the analysis for total phosphorus in either the petiole or blade tissues. When petioles of "recently matured" leaves and 2 per cent acetic acid are used, the critical phosphorus concentration is approximately 750 ppm of phosphate-phosphorus on the dry basis.

A typical potassium yield curve is given in Figure 3. It was obtained by growing sugar beet plants in culture solutions containing all necessary nutrients except potassium. By adding successive increments of potassium as potassium sulfate to the culture solution, growth increased until potassium was no longer deficient. Relating growth to the potassium concentration of petioles or of blades of "recently matured" leaves (Figure 3) indicates that the critical potassium concentration is approximately 1.0 per cent potassium on the dry basis. This value holds for blades regardless of the sodium status of the plant and for petioles only when the sodium concentrations are 1.5 per cent or above. When the sodium concentrations of the petioles are lower than 1.5 per cent, the critical level for potassium in petioles increases. Thus, in areas where sodium is apt to be low, the analysis of blades from "recently matured" leaves will give the true potassium status of the plants and is therefore preferable to the analysis of petioles as a means of estimating the potassium status of sugar beet fields. In Cali-

fornia it is unlikely that fields low in sodium will be encountered, and accordingly, petioles of leaves may be analyzed for potassium with a reasonable assurance that sodium is above the 1.5 per cent level.

The calibration curves (Figures 1, 2, and 3) may be divided into a series of zones of which the nearly horizontal portion of the curve above the critical concentration is frequently referred to as the "zone of adequacy" or of "luxury consumption." The nearly vertical leg of the curve is often called the "zone of deficiency" and the portion between these two zones is the "zone of transition." The nutrient concentration at the point of decreased growth (approximately 1,000 ppm NO_3^-–N for nitrogen, 750 ppm PO_4^-–P for phosphorus and 1 per cent for potassium) is often referred to as the "critical concentration." The critical concentration always lies within the transition zone and is quite often at the breaking point of the curve. In the zone of luxury consumption the increases in nutrient concentration do not increase yields. In the zone of deficiency the petiole nitrate-nitrogen, phosphate-phosphorus and potassium values are constant but the beet root weights increase in accordance with the amount of nitrogen, phosphorus or potassium absorbed by the beet plants. In this "zone of deficiency" the deficient nutrient is being used up by the plant as fast as it is absorbed and very little of the nutrient remains in the petioles of the recently matured leaves. High concentrations in these leaves represent "surplus" reserves not needed by the plant for growth at the time of sampling.

EFFICIENT FERTILIZATION THROUGH PLANT ANALYSIS

Once the basic information has been developed on the critical concentrations of various nutrients in plant tissues, the next step is to see how this information can be used to help fertilize crops more efficiently. Many field trials and extensive field experience indicate that plant analysis can be used to advantage in fertilizing sugar beets. Results from some of these trials are given below and will serve to illustrate various ways plant analysis may be used to develop more efficient fertilizer programs. In general these programs may be grouped as follows: 1. as a guide to current fertilization; 2. as a guide to harvest; 3. as an evaluation of current and proposed fertilizer programs and 4. diagnosis of deficiency symptoms.

Guide to nitrogen fertilization

Because it is very difficult to determine how much nitrogen a crop will require before the crop is planted, it is wise to fertilize moderately with nitrogen at planting or early thinning time. As experience has shown, most beet crops that respond at all to nitrogen can usually use at least 80 lbs of nitrogen per acre. Rates approximating this amount, therefore, are logical to start with.

Until the plants indicate a need for it, apply no nitrogen to fields

of known high fertility,

that have come out of alfalfa recently,

that have had a good legume green manure crop turned under, and/or

that have been heavily fertilized for a previous crop.

The nitrogen status of the sugar beet plants in these fields can be followed from the analysis of petiole samples. The important sampling period for deciding on a mid-season nitrogen application is from the time of thinning to mid-season when the plants are drawing heavily on the nitrogen supply in the soil. If plant analysis shows that the nitrate-nitrogen concentration is approaching dangerously close to the critical level and it is still three months or more to harvest,

Let the petioles tell the nitrogen story: Nitrate in petioles rises sharply after nitrogen fertilization but drops again as it is used up by growing plants. The information shown here tells the grower that a second 80-lb. application is ideal for an October 20 harvest. With a 40-lb. application the harvest would have to be planned earlier; with a 160-lb. application later.

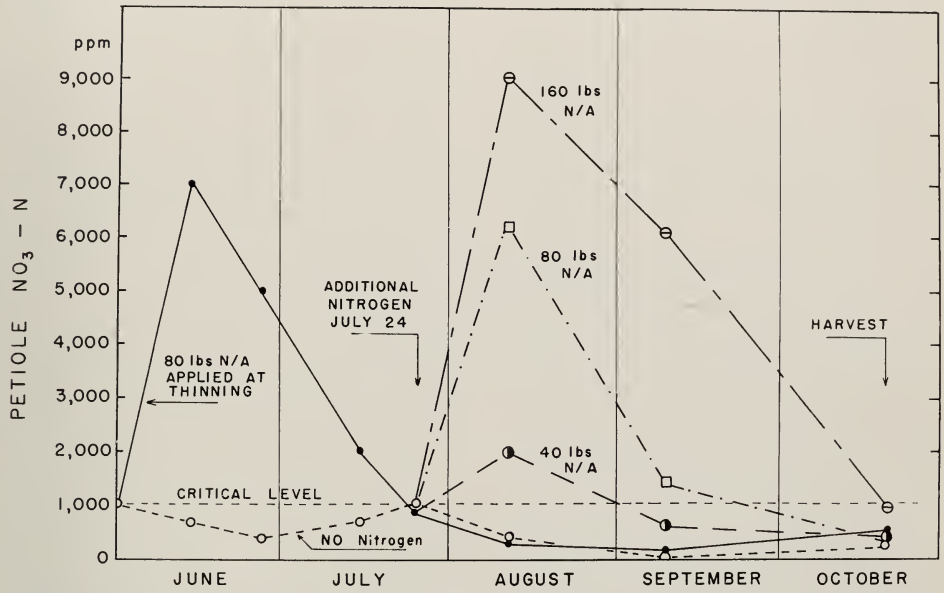

Fig. 4. Effects of fertilization on the nitrate-nitrogen content of sugar beet petioles, Davis. The first petiole samples were taken on May 31—four days after fertilization but before water had been applied to the beets. The second set of petiole samples were taken after the beets had been irrigated.

Table 1. Two 80-lbs applications of nitrogen result in greatest net return. (Harvest results, Davis.)

Lbs of nitrogen (N), phosphorus (P_2O_5) or potassium (K_2O) per acre		Ton beets per acre	Per cent sugar	Cwts sugar per acre	Net gain per acre*
First application May 27†	Second application July 24	Harvest October 20			
0	0	21.1	15.9	66.8	.....
80N	0	23.6	15.5	72.8	.....
80N	40N	25.9	15.1	78.2	$ 9.38
80N	80N	28.5	14.7	84.0	$21.17
80N	160N	30.4	14.0	84.8	$11.35
$200P_2O_5$, 80N	160N	29.7	13.6	80.6	.....
$200P_2O_5$, $200K_2O$ and 80N	160N	28.4	14.1	80.2	.....
Significant	(19:1)	2.3	0.7	10.0	.....
difference	(99:1)	3.1	0.9	13.4	.....

* Represents net gain over return from beets receiving only the first application of 80 pounds of nitrogen (Costs of the mid-season nitrogen applications as well as all differences in harvesting and hauling costs have been deducted.) Value of beets is based on a processors' net selling price of $7.00 per cwt. of sugar.

† Applied shortly after thinning.

nitrogen can be applied. A field experiment conducted at Davis will help illustrate this point. Results of this experiment are given in Figure 4 and Table 1.

Petiole samples were taken periodically from the plants, analyzed for nitrate-nitrogen and the results plotted on a graph. This is a convenient way to visualize the trend of the concentration of a nutrient in a growing crop. By referring the values so plotted to the critical concentration indicated on the graph, the nutrient status of the plants at any time can be determined.

On May 31, shortly after thinning, the first petiole samples were taken. On this date, the beets were already at the critical concentration and a nitrogen application was indicated. Phosphorus and potassium on this, and all other sampling dates, were well above the critical concentrations, and accordingly, the application of these elements was not indicated.[2]

After applying 80 lbs of nitrogen per acre, the petiole nitrate values rose sharply and then dropped back to the critical concentration at the end of July. During this time the unfertilized beets remained below the critical value and were still below when the beets were harvested on October 23.

Nitrogen was applied a second time on July 24 at the rate of 40, 80, and 160 lbs per acre, and shortly thereafter, the nitrate values were again high. The comparable plots with only the initial 80 lbs application of nitrogen, remained below the critical concentration for the rest of the season.

By taking petiole samples, the deficiency that developed at mid-season (end of July) was anticipated in time to add more nitrogen and thus improve yield and profit. Petiole samples, however, do not answer at first the question of how much more nitrogen the crop will need to produce maximum returns. This answer usually comes with experience and the use of additional petiole samples after fertilizer has been applied to see how it is used by the plants. In the example we are discussing here, the use of 40 lbs of nitrogen as the second application at mid-season increased root and sugar yields but not enough to produce maximum yields or profit. As Figure 4 shows, the second application at the 40 lb rate raised the nitrate concentration in beets above the critical level for only a short time. One hundred and sixty pounds of nitrogen applied as a second application increased root yield beyond that produced by the second 80 lbs application but resulted in a lower sugar percentage. Had the beets not been harvested until November or December, it is likely that the 160 lb application would have increased root yields still further, attained a higher sugar percentage and produced the most profitable result. In our experiment the crop was harvested in October, and the second application of 80 lbs resulted in the greatest net return (Table 1).

This and other experience indicates that when sugar beets need nitrogen at mid-season they can usually utilize a fairly large amount. Trying to "spoon feed" them with 10 to 20 lbs per acre at a time is very inefficient. Applying less than 40 lbs per acre will seldom be worthwhile.

Guide to harvest

When there is a choice of fields to be harvested, those fields that have been deficient in nitrogen the longest should be harvested first (see Fig. 4). Fields still high in nitrogen should be left unharvested until the beets become deficient in nitrogen, or other considerations make it necessary to harvest the crop. By delaying the harvest of beets high in nitrogen, the crop continues to grow and thereby beet root weights increase. If through growth the beets deplete their nitrogen

[2] As a part of the experiment, however, phosphorus and potassium in combination with nitrogen were added to other plots. The conclusions drawn from the petiole analyses that phosphorus and potassium supplies were adequate, were confirmed when the beets were harvested. Table 1.

supply, they have a better opportunity to increase in sugar content. In some instances, as shown by pot experiments conducted recently, the increase in sugar content over a four- to six-week period of nitrogen deficiency prior to harvest offset tonnage losses as great as 20 to 25 per cent. If this finding holds true under field conditions, this could lead to important savings in nitrogen, hauling and processing costs.

Evaluation of fertilizer programs

Plant analysis can be used to inventory the supply of plant nutrients available to a crop throughout a growing season. Such an inventory serves to evaluate the fertilizer program in use by seeing how well it is meeting the needs of the current crop as it is growing under actual field conditions. Three or four sampling periods are usually plenty for this purpose. These samples can indicate whether or not a crop may be suffering from deficiencies of nitrogen, phosphorus, potassium, and sulfur, and can form a basis for decisions on current fertilizer use when time permits or for use the next time the field is planted to beets or even for other crops. For example, alfalfa following beets low in phosphorus might well respond to phosphorus applications. Verification of this need might well be made through the analysis of alfalfa plant samples taken at the time of each cutting. Thus, by a systematic sampling of each crop adjustments in the fertilizer program can be anticipated and the needs of each succeeding crop met more effectively.

Diagnosis of deficiency symptoms

Whenever abnormal growth appears in a sugar beet field the grower wants to know why the plants are not growing properly. If the trouble is due to a nutritional deficiency, familiarity with the plant symptoms may be sufficient to disclose the cause of the trouble. But if the observer is not fully familiar with all the variations of a particular deficiency symptom, the diagnosis should be verified by a chemical analysis of the leaves. All yellowing is not due to a nitrogen deficiency; it could very well be due to a deficiency in sulfur, iron or zinc, or even to poor aeration or to a disease, e.g. virus yellows. Not all leaf scorching is due to potassium deficiency nor is all chlorosis associated with manganese or iron deficiency.

Directions for collecting special leaf samples are presented on pages 18 to 21 and a guide to the interpretation of the results of the chemical analysis is given in table 5.

Nutritional problems other than nitrogen

Below are examples as to how plant analysis might help in solving nutritional problems other than nitrogen.

Phosphorus. An experiment conducted in Tulare County illustrates the relationship of the concentration of phosphate-phosphorus in sugar-beet petioles to plant growth and sugar production. In this trial, nitrogen application resulted in a 7½-ton increase in beet-root weight per acre. When phosphorus was applied to beets well supplied with nitrogen, another 7½-ton increase occurred (table 2).

Figure 5 shows the effect of phosphorus fertilization on the phosphate-phosphorus content of the beet petioles. Both treatments plotted in this figure received the same amount of nitrogen (240 lbs of nitrogen per acre). The phosphate concentration in the plants that did not receive phosphorus fertilizer ranged from 440 to 680 ppm throughout the season, whereas the phosphate-fertilized plants always had a concentration of 1600 ppm or more.

The sugar beets deficient in phosphorus in this trial appeared quite normal and, in fact, produced 22 tons of roots per acre—a better-than-average crop. Such plants—growing in a field by themselves —without phosphorus fertilized beets for comparison—give very little indication that a lack of phosphorus is seriously

Let the petioles tell the phosphorus story: phosphorus fertilization kept the plants above the critical level (750 ppm—dotted line) throughout the growing season.

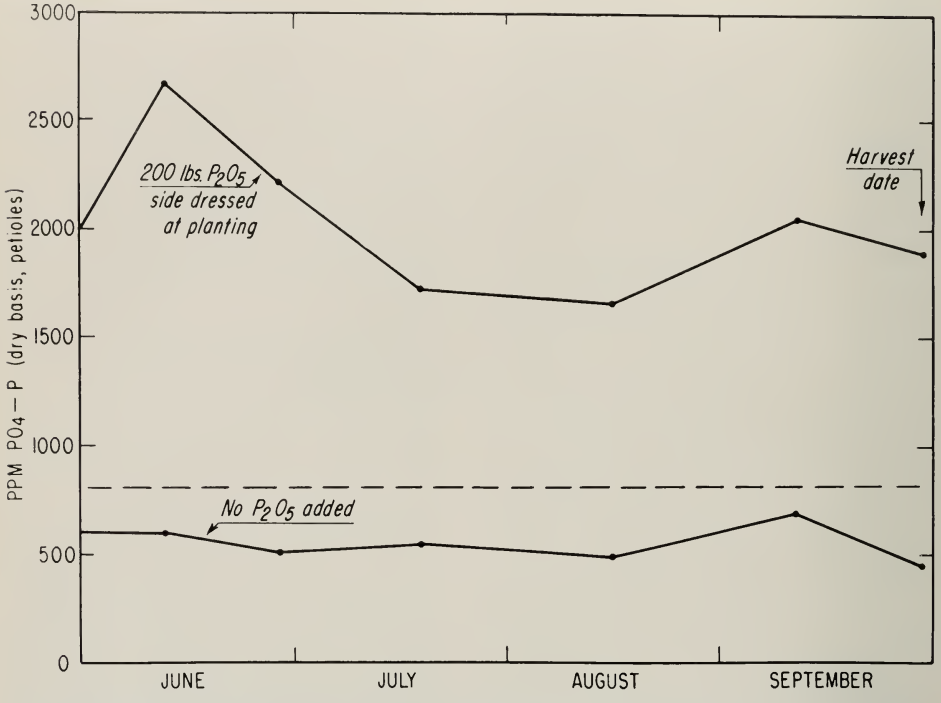

Fig. 5. The effect of phosphorus fertilization on the phosphate-phosphorus content of sugar beet petioles, Tulare County.

Table 2. Nitrogen application adds 7½ tons beets per acre. Further addition of phosphorus adds another 7½ tons per acre. (Harvest results, Tulare County.)

Lbs nitrogen (N), Phosphorus (P_2O_5) or potassium (K_2O) per acre		Tons beets per acre	Per cent sugar	Cwts sugar per acre
First application March 28*	Second application June 27	Harvest September 30		
0	0	14.5	17.8	51.4
80N	0	18.1	17.2	62.4
80N	40N	20.3	17.0	68.8
80N	80N	19.4†	15.8	61.2
80N	160N	22.0	15.3	67.4
80N, 200P_2O_5	160N	29.6	15.2	89.8
80N, 200P_2O_5, 200K_2O	160N	27.6	15.3	85.0
Significant difference	(19:1)	2.4	0.9	8.8
	(99:1)	3.2	1.2	11.8

* Planting time. Fertilizer side-dressed 6 to 8 inches from row and about 4 inches deep.
† This value is probably too low. The yield of one of the plots in this series was exceptionally low (11.5 tons/acre).

Table 3. Sugar beet leaf analysis reveals sulfur deficiency as cause of poor growth. (Leaf analysis results, Butte County, July 7.)

Growth of sugar beets	Leaf analysis (dry basis)				
	Petioles			Blades, sulfur ($SO_4^=$–S) ppm	Petioles, sulfur ($SO_4^=$–S) ppm
	Nitrogen (NO_3–N) ppm	Phosphorus (PO_4–P) ppm	Potassium (K) per cent		
Vigorous	2,520	2,120	3.15	1,880	530
Poor	9,000	2,990	3.80	155	85

limiting their growth as shown in Figure 6, below. The analysis of two or three petiole samples, however, would reveal the deficiency and would indicate the desirability of trying phosphorus on subsequent crops.

Sulfur. In Butte County a field contained a large area of yellow-colored beets having symptoms similar to those produced by nitrogen deficiency. Petiole analysis, however, showed that the yellow-appearing beets were higher in nitrate-nitrogen than the more vigorous green plants; it showed further, that there were no appreciable differences between good and poor beets in potassium and phosphorus concentrations. But when petioles and leaf blades were analyzed for sulfate-sulfur, large differences existed between good and poor beets (table 3). After these analyses had been made, ammonium sulfate, ammonium nitrate and gypsum were applied to the yellow appearing beets.

Fig. 6. The sugar beets on the left were fertilized with phosphorus, those on the right were not. Although the beets without phosphorus fertilizer produced 7½ tons of roots per acre less than the fertilized plants, they appeared quite normal all season except for slightly smaller tops.

Table 4. Sulfur-deficient sugar beet leaves (see Table 3) recover through ammonium-sulfate fertilization. Neither gypsum nor nitrogen added separately, were enough to keep plants sufficiently supplied with nitrogen and sulfur. (Leaf analysis results, Butte County, August 11.)

Material	Treatments,* Lbs per acre		Leaf analysis (dry basis)	
	Nitrogen	Sulfur	Petioles nitrogen (NO_3-N) ppm	Blades sulfur ($SO_4^=-S$) ppm
Check	0	0	640	750
Ammonium nitrate	200	0	7,600	295
Gypsum	0	484	580	13,600
Ammonium sulfate	200	242	1,350	11,000

* Applied to single 4-row strips, July 20.

The appearance of sugar beets treated with ammonium sulfate improved greatly. The deficiency symptoms disappeared, the leaves turned dark green, and there was a visible growth response. Petioles collected from these plants indicated that this growth response was associated with the absorption of sulfate-sulfur. The addition of ammonium nitrate did not correct the deficiency symptoms and the plants did not make the thrifty growth made by those receiving ammonium sulfate. When gypsum alone was added the plants readily absorbed sulfate-sulfur but made no growth response; in fact, they made less growth than plants receiving ammonium nitrate.

Reasons for these divergent growth responses can be seen in the data presented in table 4. When gypsum was added, the sulfur deficiency was readily corrected, but almost immediately the plants became deficient in nitrogen, and therefore made little or no growth response. The addition of ammonium nitrate kept the plants well supplied with nitrates but they continued to be deficient in sulfur. Ammonium sulfate corrected the sulfur deficiency and also supplied the plants with sufficient nitrogen for good growth.

Sugar-beet leaf blades appear to be a better indicator of the sulfur status of the plants than the petioles. Although there are wide differences in sulfate-sulfur concentration in deficient as compared to healthy plants in both petioles and blades, the wider range in values for the blades make them more useful as an indicator.

TAKING LEAF SAMPLES

Selection of leaf

The primary consideration in deciding what part of the plant to sample is the degree to which nutrient concentrations in a given part reflect the nutrient status of the plant. In particular, it is important to use a plant part that gives a sharp transition from a concentration reflecting a deficiency of the nutrient in question to one that indicates an adequate supply of the nutrient.

For the sugar beet, one of the youngest, fully matured leaves best meets this criterion. This type of leaf is located about mid-way between the young center leaves and leaves of the oldest leaf whorl. (See Fig. 7, page 19. The petiole of such a leaf is the best indicator of the nitrogen

Fig. 7. Selection of sugar beet leaves for analysis. Any one of the leaves marked "A" may be taken. These are recently matured and fully expanded leaves. Leaf "B" is too young and "C" is too old.

Fig. 8. Separate leaf blade from the petiole at the point indicated—where the petiole joins the blade.

and phosphorus status of the beet plant. Petioles also are satisfactory to indicate potassium status when sodium concentrations are high (1.5 per cent and above). When sodium in the plant is low, the leaf blades are superior as an indicator. For sulfur, the blade of a recently matured leaf is very suitable for analysis. In separating the petiole from the blade, the petiole should be broken where it joins the blade, as shown in Fig. 8, page 19.

Routine sampling

A typical sugar beet field is sampled in the following manner. The field is divided into approximately four equal parts. If larger than 80 acres, the field should be divided into six or eight equal parts so that each area sampled represents no more than 20 acres. The sampler walks directly across the beet rows at the midpoint of each section of the field to be sampled. One leaf is collected per plant from about 30 locations, distributed at equal intervals across each section. Thus at least four samples are collected from each field (A, B, C, and D in Fig. 9, below), each containing about 30 petioles. Unless the sampler is interested in the sulfur status of the plants, or knows the plants are high in sodium and is interested in how well the plants are supplied with potassium, the leaf blade is broken from the petiole and discarded.

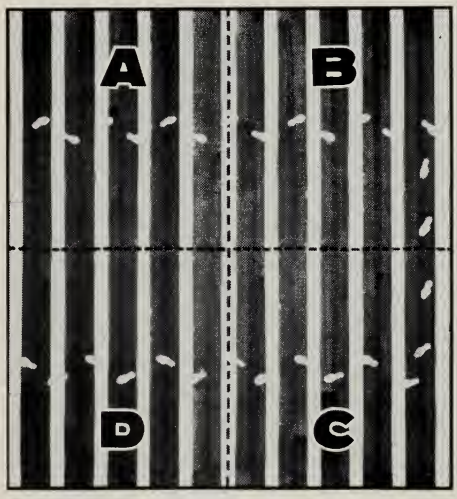

Fig. 9. Sampling a field. The sampler walks across the rows and collects four samples (A, B, C, D) of 30 petioles each.

Samples should be collected in this systematic manner unless there are areas obviously differing in plant-growth characteristics. If there are distinct growth differences, each area should be sampled independently in order to ascertain possible reasons for such differences.

Special samples

Much can be learned about abnormal growth of sugar beets within a field by collecting special samples. Where abnormal growth occurs, the sampler wants to know the reason why the plants are not growing properly. For this situation, it is desirable to collect one sample containing only material from plants exhibiting the abnormal characteristics and another from plants close by, usually in the same field, that appear normal. If a nutritional problem is involved, comparative analyses of such samples will frequently reveal the cause.

In collecting leaf samples from poorly growing plants, it is essential that the leaves be taken within a few days of the appearance of the symptoms. Otherwise secondary reactions that often take place in the mineral content of plants will obscure the real cause of the deficiency symptoms. Also, it is necessary to collect comparable plant material from normal and abnormal plants. When sugar-beet seedlings are very small, the whole top—pinched off below the cotyledons—may be collected for analysis.

Wide differences in the concentration of a nutrient in abnormal as compared to normal plants often indicates that a lack or excess of the nutrient in question is the reason for the abnormal growth. Plants can be normal, however, yet vary considerably in nutrient concentration. Table 5 has been prepared as a guide to

Table 5. A guide to critical nutrient concentrations and concentrations (dry basis) associated with and without deficiency symptoms for sugar beet plants.*

Nutrient	Plant part	Constituent determined	Critical concentration†	Range showing deficiency symptoms	Range without deficiency symptoms‡
Calcium	Petiole	Ca	..¶	0.04–0.10%	0.2–2.50%
Chlorine	Petiole	Cl	0.4%	0.01–0.04%	0.8–8.5%
Magnesium	Petiole	Mg	..¶	0.010–0.030%	0.10–0.70%
	Blade	Mg	..¶	0.025–0.050%	0.10–2.50%
Manganese	Blade	Mn	..¶	4–20 ppm	90–360 ppm
Molybdenum	Blade	Mo	..¶	0.01–0.15 ppm	0.20–20.0 ppm
Nitrogen	Petiole	NO_3^--N	1,000 ppm	0	350–35,000 ppm
Phosphorus	Petiole	$H_2PO_4^-$-P	750 ppm	150–400 ppm	750–4,000 ppm
	Blade	$H_2PO_4^-$-P	..¶	250–700 ppm	1,000–6,000 ppm
Potassium (Na more than 1.5%)	Petiole	K	1.0%	0.2–0.6%	1.0–11.0%
	Blade	K	1.0%	0.3–0.6%	1.0–6.0%
Potassium (Na less than 1.5%)	Petiole§	K	..§	0.5–2.0%	2.5–9.0%
	Blade	K	1.0%	0.4–0.5%	1.0–6.0%
Sodium	Petiole	Na	..¶	..**	0.02–9.0%
	Blade	Na	..¶	..**	0.02–3.7%
Sulfur	Blade	SO_4^--S	..¶	50–200 ppm	500–14,000 ppm

* Leaf material for chemical analysis must be collected shortly after the appearance of the leaf symptoms If this precaution is not taken, deficient plants may accumulate nutrients within the leaf without restoring "dead" or "chlorotic" tissues to a "normal" condition.

† The critical concentration is that nutrient concentration where plant growth begins to decrease in comparison with plants above the critical concentration.

‡ The upper value reported is the highest value observed to date for "normal" plants.

§ Because of the influence of sodium on the potassium content of the petioles, use the blades for potassium analysis when the petioles contain less than 1.5% sodium.

¶ Not yet determined.

** No definite symptoms observed.

concentrations of various nutrients in specific parts of sugar beet leaves that were found to be associated with certain deficiency symptoms and with normal conditions.

How often to sample

If a field is to be sampled for the purpose of applying supplemental nitrogen to a growing crop, it should be sampled at rather close intervals, especially from the time immediately after thinning to mid-season. Four or five sampling periods during this time at two-week intervals should provide the necessary information on which to base decisions as to fertilizer needs. With experience, the number of sampling periods may be reduced.

If the purpose of sampling is to determine how well a field has been fertilized and to use this information for future planning, three or four sampling periods should suffice. For this purpose, the sampling periods need not be closer than four-week intervals, but none should be separated by a period of more than six weeks. The first samples should be collected shortly after thinning, the next in early mid-season, the third in late mid-season, and the last just before harvest.

Fig. 10. Cutting petioles.

PREPARING SAMPLES

The group of petioles constituting a sample are cut into about 1/8 inch sections, using a heavy knife and chopping board, as shown in Fig. 10, above. The pieces from each sample are thoroughly mixed and one large handful placed in a bag or other suitable container and put in an oven to dry. Drying is best accomplished in a forced-draft oven at a temperature of 70–80°C (158–176°F). Drying should be completed within 12 hours without charring of the petiole pieces.

An alternative procedure for cutting the petioles prior to drying may be used when the petioles are too large to cut up easily. Draw a heavy black line across the chopping board at right angles to the operator doing the cutting. Lay the midpoint of each petiole onto the black line. Cut across the group of petioles to the right of center and cut 1/8″ sections as before. Stop cutting just left of center. With experience the correct amount of material, about 100 grams, becomes available for drying. A distinct decline in nitrate, starting from the base of the petiole to the tip, makes it essential to cut up the petioles completely or only at the mid-point as suggested if the results are to be compared to present calibration curves.

After drying, the samples are put through a grinding mill, such as the Wiley Mill, and ground to pass a 40-mesh screen. Each ground sample can then be stored in a small glass or plastic bottle. A label slipped on the inside surface of the bottle readily identifies the ground sample.

APPLYING FERTILIZER

Form of nitrogen

Field experience plus a limited number of trials using various kinds of nitrogen fertilizers indicate little or no difference in beet response to different materials when applied so as to furnish equal amounts of nitrogen. Ammonium nitrate, ammonium sulfate, calcium nitrate, so-

dium nitrate, urea, liquid ammonia and ammonia gas have all been used successfully. Generally speaking, the least expensive form of nitrogen is the most profitable one to use.

Placement of fertilizer nitrogen

Nitrogen fertilizers when applied at or before planting should not be placed too close to the row as injury may result. Side dressings at these times 6 inches away from the row and below the seed level are effective. Placement is not too critical as beet roots grow rapidly and soluble nitrogen materials can be carried to them by irrigation water or rain. In applying nitrogen fertilizers to larger beets, placement is even less critical. Applications in the center of the irrigation furrow are quite satisfactory.

Applications in irrigation water have the disadvantage that the nitrogen distribution will be no more uniform than the distribution of irrigation water.

The effectiveness of nitrogen fertilizers is closely related to water movement in the soil. It usually takes an irrigation or a rain to disperse nitrogen throughout the soil for maximum effectiveness. Irrigation should follow nitrogen applications as soon as practical. Nitrate-nitrogen is easily leached so irrigation water should not be applied in excess of that required to replenish the soil moisture in the root zone. Movement of nitrate with water into the soil makes this form of nitrogen useful in mid-season applications when a delay in availability is undesirable.

Phosphorus

In contrast to nitrogen fertilizers, phosphate materials move very slowly from where they are placed in the soil. Also, certain soils have the ability to fix phosphates in a form not available to plants. For these reasons, best results are usually obtained when phosphate materials are placed in the effective root zone. Side dressing phosphate materials in the shoulder of beet beds has been satisfactory in most areas in California. On certain soils of the Sacramento Delta, however, where phosphate deficiencies are extreme, a placement at planting time of 40 to 50 pounds of P_2O_5 per acre in close proximity to the seed is recommended. This amount of P_2O_5 from treble-superphosphate placed an inch or so below the seed has been very effective in overcoming early season phosphate deficiencies. Later in the season sugar beet plants, because of soil drying, fail to absorb phosphorus even when it is placed at a depth of six to eight inches.

Potassium

Potassium may be applied as the sulfate or chloride salt as a side dressing at thinning time. Two hundred pounds of K_2O has been effective in field experiments in increasing the potassium content of petioles. Where potassium deficiencies are expected, it would be well to try this rate in strips in the field. If a growth response establishes the existence of a deficiency, an additional trial may be run to determine the optimum rate.

Foliar applications

Attempts have been made to apply foliar sprays containing nitrogen. Such sprays have the disadvantage that only small amounts of nitrogen can be applied without burning the leaves. Also, leaf applications do not appear to be much more efficient than soil applications and beets require about the same amount by either method for maximum growth. Not much more than 20 lbs of nitrogen per acre has been applied as a leaf spray without seriously burning the leaves. When sugar beets are deficient in nitrogen, it usually takes at least 40 lbs per acre, and more often 80, to produce maximum returns. Applying this amount by foliar sprays would take several applications.

ACKNOWLEDGMENT

The authors thank the many University of California Farm Advisors and sugar company field men who have helped in the field research mentioned in this bulletin. The following Farm Advisors in particular have contributed much to an understanding of how research on plant analysis might be used in a practical way on farms in California: Harwood L. Hall, David M. Holmberg, Torrey Lyons, William S. Seyman, Robert A. Brendler, Robert S. Ayres, William R. Sallee.

II. Analytical Methods For Use in Plant Analysis

CONTENTS

	Page
Determination of macronutrient elements	27
Ashing methods	27
Dry ashing	28
Dry ashing with sulfuric acid	28
Removal of silica from dry ashes	29
Dry ashing with the addition of basic solutions	29
Wet ashing	29
Apparatus for wet digestions	30
Reagents needed for wet oxidations	31
Ternary acid wet oxidation procedure	31
Wet oxidation with nitric and perchloric acid	32
Calcium by the oxalate method	33
Calcium and magnesium by the EDTA method	35
Chloride by the Mohr method	38
Chloride by potentiometric titration	39
Magnesium by the thiazole yellow method	41
Nitrate by the diphenylamine test	43
Nitrate nitrogen in the presence of excess chloride	44
Nitrate nitrogen in the absence of excess chloride	45
Potassium by the cobaltinitrite method	45
Potassium and sodium by flame photometer	47
Phosphorus—acid soluble	49
Phosphorus—total	52
Sulfur (sulfate) by the methylene blue method	54
Sulfur (total) by the methylene blue method	57
Determination of the micronutrient elements	58
Collection and preparation of samples	58
Glassware and reagents for micronutrient analysis	59
Dithizone as a reagent for micronutrients	60
Boron	62
Copper	64
Iron	66
Manganese	67
Molybdenum	68
Zinc by the Zincon method	71
Zinc by the dithizone method	74
Literature cited	78

II. ANALYTICAL METHODS

For Use in Plant Analysis

CLARENCE M. JOHNSON ALBERT ULRICH

THIS part of the bulletin describes suitable methods for the quantitative determination of many of the elements of importance in the assessment of the nutrient status of plants. It is not intended to be a textbook of analytical methods but it is hoped that the description of the methods are sufficiently detailed that competent chemists will be able to obtain accurate and reliable results.

The methods described here are either those devised by the authors and their colleagues or are modifications of methods recorded in the literature. In all cases the methods are based on sound chemical principles with the necessary modifications made to take into account interferences that may be peculiar to plant materials or to increase the sensitivity of the method to permit the determination of elements in the microgram rather than milligram range. Methods of ashing or solubilizing the sample often require special attention to avoid losses either by volatilization or occlusion of the element. Often extremely small amounts of the element have to be separated from the ash residue of several grams of sample. At the risk of being repetitious, some of these details have been discussed in each section dealing with the determination of each element.

The methods described are not the only ones that have been used or proposed by agricultural chemists. They have been used by the authors, and found to provide the degree of accuracy and precision necessary for the solution of nutritional problems. Every effort has been made to achieve simplicity of procedure without sacrificing accuracy and specificity. While the methods may be considered to be "laboratory tested" it must be realized that trustworthy results may be obtained only by strict adherence to details as outlined in the methods. On the other hand, occasions may arise where difficulties are encountered not discussed in this presentation. In spite of apparent simplicity of a method every analyst realizes that experience is necessary to develop real proficiency in the determination of any element.

It is essential that blank determinations and standards be run with each lot of samples, if the chemist is to report his analytical results with confidence. As a further check on the performance of the method and the analyst as well, it is useful to carry along with the unknowns an actual plant sample whose content of the element has been established by many analyses.

Analytical results are always expressed in terms of concentration of the *element* regardless of the form determined. For example, in the nitrate method, soluble *nitrate* (excluding organically combined nitrogen and ammonia) is determined but the results are calculated in terms of nitrogen (N) rather than as nitrate (NO_3^-). Thus the result given as 1000 ppm nitrate-nitrogen (NO_3^-–N) means that each gram of sam-

ple contained 1000 micrograms (or 1000 milligrams per kilogram) of nitrogen (N) in the nitrate form. Similar considerations hold for determination and reporting of phosphate-phosphorus and sulfate-sulfur. Cation analyses are reported in terms of the element, not as the oxide.

Equipment required for the determinations is for the most part conventional. Special equipment will be noted when required. Particular attention is called to procedures involving measurements of intensity of color. For many determinations a filter type photo-electric colorimeter is satisfactory although in all cases better precision, sensitivity, and greater freedom from interferences will be obtained with a spectrophotometer using a grating or prism to effect spectral dispersion and isolation. The effective length of the light path through the colored solution, of course, determines the ultimate sensitivity and also the range of amounts of the element that may be determined in a procedure. Some colorimeters are provided with solution cells that have an effective light path distance of 40 mm while others have light paths of only 10 mm. Obviously the longer light paths permit quantitative estimation of much smaller amounts of a colored substance in a given volume of solution. However, the cells with the longer light paths usually require quite large volumes of solution, up to 50 ml while many of the cells with 10 mm light paths require only 5 ml of colored solution. Often a colorimetric method may be scaled down to take advantage of the smaller required volume thus effectively increasing sensitivity to the point where a 10 mm light path may be fully as sensitive as the 40 mm path. If a method is scaled down, care must be taken to maintain all reagent concentrations at the same level as that used in the original method. Blanks and standards must be run with the samples.

DETERMINATION OF THE MACRONUTRIENT ELEMENTS

Procedures are described in this section for the determination of calcium, magnesium, potassium, sodium, phosphate-phosphorus, total phosphorus, sulfate-sulfur, total sulfur, and nitrate-nitrogen. Sodium, although not regarded as an essential nutrient element for plants, is included in this group because of its interaction with potassium in the nutrition of some plants and because it can be determined simultaneously with potassium with little extra work.

The determination of phosphorus as phosphate-phosphorus and sulfur as sulfate-sulfur has been found, thus far, to have the greatest use in the diagnosis of the nutrient requirements of plants for these elements. Primarily for this reason, methods have been devised for the determination of these ions rather than for total element analysis, which includes both inorganic and organic forms of these elements. Nevertheless, a description of the methods for total phosphorus and sulfur are included, since the separation of the organic and inorganic forms is desirable in certain animal and plant nutrition studies.

Ashing Methods

There are two ashing methods, each having many variations of technique, and each having its merits and disadvantages. Often personal considerations and availability of equipment and reagents may determine whether the sample may be dry ashed or wet ashed. In a few instances the use of a particular method is mandatory because in alternative methods a partial or complete loss of an element occurs. For example, boron is completely lost when the sample is prepared by wet digestion and dry ashing is required; but for some micronutrients considerable

time may be saved if the ashing is done by the wet method thus eliminating the need for treatment of the dry ash with hydrofluoric acid to remove silica. Descriptions of methods of determination of each element will include a recommendation for ashing procedure.

Dry ashing

Dry ashing may be done on the plant material alone or, for special reasons, with the addition of other reagents prior to burning in the muffle furnace.

Dry ashing with sulfuric acid. Dry ashing for sodium, potassium, calcium, and magnesium is most conveniently done after the addition of a small amount of sulfuric acid. The sulfated ash so obtained has less tendency to fuse to the dish and thus prevents loss of some of the elements. Sulfuric acid is most easily added to the sample in the ashing dish (porcelain or platinum) by adding sufficient 5 per cent sulfuric acid in ethyl alcohol (50 ml concentrated sulfuric acid added to 950 ml 95 per cent ethanol) to saturate the plant material. The alcoholic solution of sulfuric acid readily permeates the whole mass of plant material without the local carbonization that often results when concentrated sulfuric acid is added directly. The use of the alcoholic solution has an advantage over the use of a dilute aqueous solution of sulfuric acid in that the excess solvent may be quickly removed by burning rather than by a long evaporation period. The excess alcohol in the dishes should not be ignited with a match because of the danger of introducing potassium from the ash of the match; rather, the pipet used for dispensing the alcoholic sulfuric acid is used as a torch.

After the excess alcohol has burned off, place the dish in a cold electric muffle and heat to 500–550°C. The rate of heating should not be too rapid, because of possible loss of ash if the sample bursts into flame or glows too violently. The dishes should not be set directly on the floor of the muffle because of danger of localized overheating with possible fusion of the ash in the dish. A false floor of one or two layers of nichrome gauze is useful. Likewise nichrome gauze placed on top of the dishes may serve as support for one or more layers of dishes. If the muffle furnace does not provide uniform temperature conditions, it may be necessary to change positions of the samples as the ashing proceeds. Sample sizes to be taken for the ashing depend on the amount of material required for the analysis but dishes should be large enough in diameter to allow spreading of the sample in a thin layer to permit uniform access of air to all the material or else the carbon formed in the lower portion of the sample will be difficult to oxidize completely. Large amounts of carbon should be regarded with suspicion and it may be necessary to filter the ash and return unashed carbon and the filter paper to the muffle for complete ashing. Ordinarily, there is no value in obtaining the weight of the ash.

After the ash appears to be free of carbon, showing only a grey color, remove the dishes from the muffle and allow to cool. Moisten the ash with a small amount of water and cautiously add dilute (3 N) hydrochloric acid until the ash solution is distinctly acid and warm on the steam bath until all salts except silica are in solution. For some analyses, as noted in individual procedures, it is only necessary to filter the silica off.

In the authors' laboratories dry ashing is employed for potassium, sodium, calcium and magnesium analyses. Small porcelain dishes (Coors No. 000) are used. A large muffle furnace, inside dimensions of 18″ long, 10″ wide and 6″ high, will accommodate at least 40 dishes —20 on each layer. Generally, there is no need to remove silica from the ash except by filtration prior to analyses for potassium, sodium, calcium and magnesium. Once the 40 samples are placed in the muffle furnace and the heating started

no more attention is required and the ashing time per sample is very small indeed. This would not be the case if it were necessary to do the ashing in platinum ware, followed by a time consuming hydrofluoric acid treatment.

Removal of silica from dry ashes. Silica removal from dry ashes is required for exact determination of copper, iron, manganese, molybdenum and zinc. Ashing must be done in platinum dishes. Moisten the cool ash with a little glass-distilled water, add 5 drops of concentrated sulfuric acid and 5 ml of 48 per cent hydrofluoric acid. (**Caution:** Do not allow hydrofluoric acid to contact the skin. The effect of hydrofluoric acid on the skin is not felt at once and by the time the discomfort is felt, it is difficult to remove the acid. It is therefore good practice to always wash the hands immediately after handling the acid even if it does not seem likely that any acid has been spilled.) Volatize the silica by evaporating the solution gently to dryness on a hot plate. When dry, return the samples to the muffle furnace at 500°C for 10 minutes to remove last traces of carbon. Treat this residue again with 5 drops of concentrated sulfuric acid, 5 drops of hydrofluoric acid and 2 ml of glass-distilled water. Fume off the water and hydrofluoric acid on a hot plate but do not fume off all of the sulfuric acid. Take up the residue in 10 ml of 3 N hydrochloric acid and 20 ml of glass distilled water. Warm on the steambath to aid in dissolving calcium sulfate. If difficulty is encountered in dissolving calcium sulfate, add more hydrochloric acid. Transfer the solution and washings to a volumetric flask, make to volume and mix.

Dry ashing with the addition of basic solutions. This procedure may be used for special analyses as, for example, for total phosphorus, sulfur, boron and chlorine. However, most analysts prefer wet ashings for total phosphorus and sulfur. Addition of base to samples for boron determination is unnecessary except in cases where there is a large excess of anionic substances over cationic as in seeds and other fatty materials. Added base is required for retention of chloride in all cases where the sample is ashed. The method for chloride to be described later, however, does not require ashing because it has been shown that, except where chloride is extremely low, equally good results may be obtained from titration of an aqueous extract of the plant material.

Basic materials added to plant samples include magnesium nitrate, magnesium acetate, calcium hydroxide, potassium carbonate and sodium hydroxide. Magnesium nitrate addition often leads to deflagration of the sample with possible loss of ash constituents and magnesium acetate is usually preferred if phosphorus is to be determined on dry ashings of plant material. Considerable difficulty is encountered in obtaining a carbon-free ash when calcium hydroxide, potassium carbonate or sodium hydroxide is added to plant material for ashing. It is usually necessary to filter the solution of the ash and reignite the paper and carbon residue. None of the methods to be described here requires the use of alkaline additives in ashing. If it is felt necessary to use such additives, the reader should refer to appropriate sections in the "Official Methods of Analysis" of the Association of Official Agricultural Chemists (1955)[3] or to "Soil and Plant Analysis" by C. S. Piper (1953).

Wet ashing

Wet ashing of plant materials is eminently superior to dry ashing for the determination of all elements (except for boron and chlorine) for these two reasons:

(1) There is no danger of loss of the element (save for boron and halides) because the temperature of the digest cannot exceed the boiling point of the sulfuric

[3] See "Literature Cited" for citations referred to in the text by author and date.

or perchloric acids, 338°C for sulfuric acid and 203°C for perchloric acid.

(2) The silica formed in the wet digest is completely dehydrated and left in a form where adsorption of micronutrients is negligible. Silica resulting from dry ashing will adsorb appreciable and variable amounts of the micronutrient elements and must be removed by volatilization with hydrofluoric acid. This latter step is time consuming and requires the use of platinum dishes for ashing.

There are some disadvantages in wet ashing procedures. First, substantially greater amounts of reagents are required; Second, the analyst's time per sample is somewhat high; and third, provision must be made for disposal of considerable volumes of acid fumes. Fume disposal systems similar to those required for Kjeldahl digestions are satisfactory but the fume pipe must be made of borosilicate glass rather than lead because of the danger of contamination with heavy metals from the lead. The glass fume pipe should be so constructed that it can be easily washed. Jackson (1958, page 332) describes and illustrates a very useful and simple apparatus for disposal of fumes from wet digestions. *Under no circumstances should perchloric acid fumes be vented directly into a fume hood.* Perchloric acid fumes may condense on walls of the hoods and in the fume ducts and cause fires and explosions under some conditions. The perchloric acid and other acid fumes must be disposed of by absorbing in water or sodium hydroxide and washing down the drain.

Apparatus for Wet Digestions. *Micro-Kjeldahl digestion rack with glass fume manifold.* Lead fume pipes must

Hazards and Precautions in the Use of Perchloric Acid

Perchloric acid is an extremely useful reagent in the wet digestion of plant materials. Failure to understand its properties and reactions may lead to fires and explosions but there is no justification for the unreasoning fear of perchlorate digestions that is encountered in some quarters. Hundreds and thousands of wet digestions with perchloric acid have been done in the authors' laboratories without fires or explosions. Before undertaking wet digestions with perchloric acid, the chemist should become familiar with the properties of perchloric acid and the few simple precautions required for the successful use of the reagent.

At room temperature, perchloric acid, both dilute and concentrated, behaves as a strong acid only and has no oxidizing power. As the temperature of the solution is increased, water is boiled off until the perchloric acid becomes more and more concentrated and finally perchloric acid dihydrate ($HClO_4 \cdot 2H_2O$) forms a constant boiling mixture. The dihydrate boils off with no increase in temperature from the constant boiling temperature of 203°C. This hot constant boiling mixture is one of the most powerful oxidizing agents known and it is also a powerful dehydrating agent. FOR THIS REASON OXIDIZABLE SUBSTANCES SUCH AS PLANT MATERIAL, ORGANIC SOLVENTS, FILTER PAPER, ETC. SHOULD NEVER BE ALLOWED TO COME IN CONTACT WITH HOT PERCHLORIC ACID WITHOUT A PREOXIDATION WITH NITRIC ACID. EXPLOSIONS AND FIRES ARE BOUND TO RESULT IF THIS PRECAUTION IS NOT OBSERVED. DISTILLATION OF PERCHLORIC ACID, FOR PURIFICATION FROM HEAVY METALS, SHOULD NOT BE ATTEMPTED.

Pretreatment of plant materials with concentrated nitric acid as described below renders the wet digestion procedure perfectly safe. Easily oxidizable compounds such as ethyl alcohol, glycerine, and ether should never be allowed to come into the digestion mixture.

not be used when micronutrients are to be determined because lead contamination interferes in many of the determinations. The holes in the transite shelf of the unit should not exceed 7/8" in diameter and the flame should not be high enough to permit undue heating of the glass above the liquid level. If necessary provide additional shielding with small circles of asbestos paper. Free flame heating of the glass surface above the liquid level may lead to explosive decomposition of perchloric acid or ammonium perchlorate. The fume manifold should be connected to a glass water pump to provide for disposal of fumes to the drain. Fumes should not be discharged directly into the fume hood because of their corrosive effect on the hood and ducts as well as the possible fire and explosion danger of condensed perchloric acid fumes in the hood and duct system.

Micro-Kjeldahl flasks, 30 ml. Samples up to and including 1 gram may be conveniently digested in flasks of this size. Samples up to 3 grams in weight may be digested in 100 ml Kjeldahl flasks in the micro digestion rack.

Macro-Kjeldahl digestion rack. This rack should be equipped with a glass fume manifold because of the danger of contamination from lead fume pipes which may lead to erroneous results in analyses for micronutrients. As noted above, special precautions must be taken to discharge the acid fumes to the drain rather than to the hood system. The macro-digestion unit is used for samples larger than 1–3 grams as for example in wet digestion for the macronutrients or where a large sample is digested, made to volume and appropriate aliquots taken for an analyses for several nutrients.

The wet oxidation unit described by Jackson (1958) appears to be a very useful apparatus. This unit is so designed that conical flasks of different sizes may be used. Provision is made for disposal of acid fumes through a sodium hydroxide trap and glass water pump system.

Reagents Needed for Wet Oxidations. *Concentrated nitric acid.* ACS grade.

Ternary acid mixture. Mix 10 volumes of concentrated nitric acid, 1 volume of concentrated sulfuric acid and 3 volumes of 72 per cent perchloric acid. Mix in order given and allow to cool before use.

Normally these acids contain only negligible amounts of the micronutrients that are to be determined but blanks containing the same amounts of acids as used with the samples should be run at frequent intervals and appropriate corrections applied to the readings obtained on the samples.

Bumping is frequently a problem in wet oxidations. A single Hengar granule is very effective in preventing bumping. Glass beads are less effective.

Ternary Acid Wet Oxidation Procedure. *Predigestion with concentrated nitric acid.*

Transfer samples to digestion flask. Samples up to and including 1 gram may be digested in 30 ml micro-Kjeldahl flasks. Large samples should be done in larger Kjeldahl flasks or Erlenmeyer flasks. Add a Hengar granule and *5 ml* of concentrated nitric acid for *each gram* of dry sample. Swirl to wet all the sample and allow the samples to stand at this stage overnight, after which they may be heated with little or no danger of foaming over. If it is desired to proceed with the digestion at once, heat carefully on the steambath or over the flame or low electric heater, paying particular attention to foaming. It may be necessary to cool the flask by immersion in cool water to prevent loss of sample by foaming. When foaming has ceased, increase the rate of heating but do not apply so much heat that copious nitric oxide fumes are lost. If nitric acid is lost too rapidly, acid is wasted and the oxidation is not carried as far as it should be. When solid matter is in solution and the volume of the nitric acid has reduced to less than half the original volume, cool the digest and

add 5 *ml* of the ternary acid mixture for *each gram* of sample. Resume heating. Boiling at this stage can be quite vigorous. The nitric acid in the early stages is the only effective oxidizing agent. It is not until most of the nitric acid has been driven off that the temperature and concentration of perchloric acid is high enough to render the perchloric acid effective in oxidizing the difficultly oxidizable material. Normally, the increase in temperature and concentration is sufficiently gradual that the oxidation proceeds in a controlled manner. With large samples, the digest may turn brown or black for a minute or two as the perchloric acid is reacting. However, the digest quickly becomes clear and the boiling rate should be maintained unless the material in the flask appears to be going dry. If the digest appears to be on the verge of turning to a solid black mass, stop the heating, cool the flask and add another 5 ml of the acid mixture to the flask and resume heating. This last step rarely is necessary unless an error has been made in calculating the amount of acid to be added after the predigestion or unless the sample was of exceptionally fatty nature, as for example seeds.

Continue the digestion of the clear solution until all or almost all of the perchloric acid has been volatilized leaving only a salt residue in the small volume of sulfuric acid. Cool the digest and wash down the neck of the flask with about 5 ml of glass distilled water and heat again to fumes of sulfuric acid. Cool the digest, add 5–10 ml of glass distilled water and heat to near boiling for about 5 minutes to insure solution of salts. If the sample was extraordinarily high in calcium, an insoluble residue of calcium sulfate may remain. Addition of an equal volume of concentrated hydrochloric acid often aids in the solution of the calcium sulfate. Presence of hydrochloric acid is undesirable in the determination of manganese and in this case it is best to filter and wash the calcium sulfate precipitate. If the amount of the precipitate is small, it suffices to merely allow the precipitate to settle to the bottom of the volumetric flask before taking aliquots for determinations. The calcium sulfate precipitate formed in the highly acid solution does not appear to adsorb significant amounts of the micronutrients. Obviously, if calcium is to be determined on the sample, all the precipitate must be dissolved.

Other precipitates may be found in the acid digest. Acid digests of soil grown plants invariably have a residue of silica which may be distinguished from other insoluble residues by its fine granular appearance. Fortunately, silica residues resulting from wet digestions, as contrasted to those from dry ashings, do not adsorb more than traces of micronutrients and can be disregarded. If the sample contained large amounts of potassium and the removal of perchloric acid was incomplete, a precipitate of potassium perchlorate may be found when the digest cools. Potassium perchlorate forms well defined crystals in the cooled solution. Another distinguishing feature of potassium perchlorate precipitates is the high temperature coefficient of solubility. Frequently the solution in the volumetric flask may be warmed slightly and the crystalline precipitate will dissolve. The contents of the flask can then be mixed again, cooled to room temperature and the aliquot taken before the precipitate forms again (the solution tends to become supersaturated with respect to the potassium perchlorate).

Wet Oxidation with Nitric Acid and Perchloric Acid. The wet oxidation using sulfuric, nitric and perchloric acids together is the procedure most generally recommended, especially for large samples. However, there are special cases where the introduction of sulfuric acid is undesirable as for example in the wet ashing procedure for total sulfur. In many other cases equally good ashing results can be obtained from the use of nitric and perchloric acids alone and thus

avoiding the troublesome calcium sulfate precipitate.

Sample sizes are generally limited to 1 gram when the oxidation is carried out without sulfuric acid although samples as large as 3 grams have been routinely digested in the authors' laboratories without difficulty. Place the sample and a Hengar granule in a Kjeldahl flask (30 ml flask for 1 gram, 100 ml flask for samples up to 3 grams) and add *10 ml* of concentrated nitric acid for *each gram* of sample. Swirl the flask in order to wet the dry material with the nitric acid. Allow the sample to stand overnight at room temperature if convenient. If it is desirable to proceed with the digestion at once, place on the steam bath taking care to avoid loss of the sample from the flask from foaming. It may be necessary to cool the flask if the foam becomes excessive. When the initial foaming has ceased, heat the flask gently to boiling over a microburner. The rate of heating should be so regulated that large amounts of nitric oxide fumes are not lost. When all the solid matter is in solution and the volume of the nitric acid is reduced to about half its original volume, cool the digest and add *2 ml* of perchloric acid for *each gram* of sample and resume heating. After most of the nitric acid has been driven off, the digest will have only a pale yellow color. The temperature of the liquid increases as the nitric acid is boiled off until finally the perchloric acid oxidizes the remaining organic matter and the digest frequently will turn brown to black and a vigorous reaction sets in and lasts for a minute or two. The digest then clears but the heating should be continued for at least one half hour after the dense white fumes of perchloric acid appear in the flask. The heat should be regulated so that the surface of the flask above the liquid does not become too hot. In addition to regulation of flame size this may be accomplished by shielding the exposed portion of the flask with small pieces of asbestos. Excessive heating of the flask above the liquid level may result in loss of sulfuric or phosphoric acids resulting in low analytical values for sulfur and phosphorus. If this precaution is observed and if the volume of perchloric acid is not reduced below one or two ml, recovery of sulfur and phosphorus is complete.

If the sample contains large amounts of potassium, more than 3 or 4 per cent, a precipitate of potassium perchlorate may form and tend to cake on the bottom of the flask. Care should be taken to rotate the flask to prevent caking. This precipitate dissolves in water when the digest is made to volume. If the potassium content is extremely high, some of the potassium perchlorate may not stay in solution at room temperature. It may be necessary to warm the solution slightly before taking the aliquot for analysis.

Special directions for wet oxidation of plant samples for total sulfur determination are given in the section on sulfur determination.

Calcium by the Oxalate Method

Calcium concentrations in various plant tissues range from 0.02 per cent to 5.0 per cent.

Calcium is determined in plant ash with a minimum of interference from other plant ash constituents by precipitation and separation of calcium oxalate, followed by titration of the oxalate with standard permanganate. The procedure is so designed that reagents are added in a routine manner without need for special pH indicators or preliminary separations. From 30 to 40 samples may be analyzed conveniently during an eight hour day. The standard deviation of a single determination is $\pm$ 0.02 or about 1 per cent of the mean for a sample containing 1.92 per cent calcium (on a dry basis).

Reagents

Alcoholic sulfuric acid. Add 50 ml of concentrated sulfuric acid to 950 ml of 95 per cent ethanol.

Solution A. Dissolve 27 grams of ammonium chloride in water, add 86 ml of concentrated hydrochloric acid and dilute to 2 liters.

Solution B. Dissolve 50 grams of oxalic acid $(COOH)_2 2H_2O$ and 70 grams of ammonium oxalate $(NH_4)_2C_2O_4H_2O$ in water and dilute to 2 liters.

Solution C. Dissolve 400 grams sodium acetate trihydrate $(NaC_2H_3O_2 \cdot 3H_2O)$ in water and dilute to 2 liters.

Standard calcium solution. Dissolve 6.292 grams of mono-calcium phosphate mono-hydrate $(Ca(H_2PO_4)_2H_2O)$ in water in a 1 liter volumetric flask, make to the mark and mix. One milliliter of this solution contains 1 milligram of calcium.

Sodium oxalate standard solution, 0.05 N. Dissolve 3.350 grams of anhydrous sodium oxalate $(Na_2C_2O_4)$ in water, add 10 ml of concentrated sulfuric acid and make to volume in a 1 liter volumetric flask.

Potassium permanganate solution, 0.05 N approximately. Dissolve 3.2 grams of potassium permanganate $(KMnO_4)$ in 2 liters of distilled water in a large beaker or boiling flask. Boil gently for 10 to 15 minutes to oxidize reducing substances and precipitate oxides of manganese, cool overnight and filter through a sintered glass filter. Protect this solution from dust and reducing fumes. Make to 2 liter volume. Standardize against the standard sodium oxalate solution at frequent intervals.

Sulfuric acid, 1:1. Add concentrated sulfuric acid to an equal volume of distilled water.

Procedure

Transfer ground plant sample to porcelain evaporating dishes (Coors 000). For most plant materials, a 0.300 gram sample is convenient. Add 2 ml of alcoholic sulfuric acid to the material and after allowing time for the alcoholic sulfuric acid to permeate the sample, ignite the alcohol. Ash the samples in a muffle furnace at 660°C for 3 to 4 hours or until a white, carbon free ash is obtained. Ashing overnight at 550 to 600°C is also permissible. Moisten the cool ash with distilled water and add 5 ml of solution A (ammonium chloride-hydrochloric acid), transfer the ash solution to 100 ml beakers, using boiling distilled water to wash the dishes. The total volume of the ash solution and washings should be approximately 40 to 50 ml. Bring the solution to a boil and add 5 ml of solution B (ammonium oxalate-oxalic acid). Add 4 ml of solution C (sodium acetate). The pH of the final solution will be automatically adjusted to 4.3 ± 0.2 after adding solutions A, B and C in the proportions given above. The pH is not extremely critical provided it lies between 3.8 and 4.5. Lower pH conditions may result in incomplete precipitation of calcium oxalate, higher ones are conducive to coprecipitation of other ions.

Cool the solutions, allow to stand at least one hour after cooling to room temperature and filter through sintered glass filters provided with a filter aid mat, or through a Gooch crucible with an asbestos mat. The solutions may be allowed to stand overnight before filtering, although this is not necessary and in fact may lead to slightly high results from post-precipitation of magnesium.

Refer to directions given in the cobaltinitrite procedure for potassium for details of preparation of celite, or talc, filter aid. The amount of filter aid to be used will depend on the porosity of the sintered glass funnels and the particle size of the filter aid. Too little filter aid may result in incomplete recovery of calcium oxalate; too much filter aid unnecessarily increases the time required for filtration and washing.

The filter aid and precipitate should be washed with several small portions of cold distilled water. Under no circumstances should hot water be used because of the large solubility of calcium oxalate.

Best results are obtained by washing with distilled water which is ice cold.

Wash the precipitate and filter aid with hot water into 100 ml beakers. Final volume after washing will be approximately 50 ml. Add 5 ml of the 1:1 sulfuric acid and bring nearly to boiling point. A thermometer may be used as a stirring rod, also permitting the operator to be certain of maintaining the temperature in the range of 80 to 90°C during the titration. Titrate the oxalate with 0.05 N potassium permanganate. Add the first few drops slowly, allowing each drop to be decolorized before adding more. After several drops have been added, the remainder of the titration may be carried out with normal speed to a faint permanent pink color of excess permanganate.

Standard calcium solutions should be carried through the precipitation procedure at frequent intervals to check on the efficiency of precipitation and washing of the precipitate. Convenient amounts of calcium to be taken through the procedure are 2, 5 and 10 milligrams.

One milliliter of 0.05 N potassium permanganate is equivalent to 1 milligram of calcium.

Calculation

$$\frac{\text{Milliliters 0.05 N permanganate required}}{\text{Sample weight in milligrams}} \times 100 = \text{per cent calcium in sample}$$

Calcium and Magnesium by the EDTA Method

The determination of calcium and magnesium in plant material is subject to more interferences than normally encountered in similar determinations in soil extracts and waters. Iron, manganese, copper, and zinc interfere by reacting with the indicators but the effects of these elements may be effectively circumvented by the addition of cyanide ion and triethanolamine to the solution prior to adding the indicator. Phosphate interferes by precipitating calcium and magnesium phosphates under the alkaline conditions required for the titration. Phosphate interference is overcome by adding a slight excess of the EDTA solution over that required to give a stable endpoint with several minutes of continuous stirring. The EDTA chelates of calcium and magnesium are more stable than the corresponding phosphates and will slowly dissolve the precipitates. The excess EDTA is back titrated with standard calcium solution followed by exact titration with EDTA to the final endpoint.

Equipment

Microburets. 10 ml graduated at intervals of 0.02 ml.

Adjustable light source. Ordinary gooseneck type desk lamp is suitable.

Reagents

Sodium hydroxide-sodium cyanide solution. Dissolve 80 grams of sodium hydroxide in 1 liter of distilled water. To the cooled solution add 10 grams of sodium cyanide.

Ammonium chloride-ammonium hydroxide buffer solution. Dissolve 67.5 grams of ammonium chloride in 570 ml of concentrated ammonium hydroxide. Add 10 grams of sodium cyanide and dilute to 1 liter with distilled water.

Standard calcium solution. 0.01 N. Dissolve 0.500 grams of pure calcium carbonate in 30 ml of 1 N HCl and dilute to 1 liter with distilled water. One milliliter of this solution contains 0.01 milliequivalents or 0.20 milligrams of calcium.

Calcein indicator solution. Dissolve 0.2 gram of Calcein in 25 ml of 0.1 N sodium hydroxide and dilute to 100 ml.

Eriochrome Black T indicator. Dissolve 0.5 gram of Eriochrome Black T. and 4.5 grams of hydroxylamine hydrochloride (H_2NOH HCl) in 100 ml of 95 per cent ethyl alcohol. Prepare fresh solution at monthly intervals.

EDTA solution. 0.01 N. Dissolve 1.9 grams of disodium dihydrogen ethylenediamine tetraacetate dihydrate (Molecular weight 372) in distilled water and dilute to 1 liter. This solution is standardized against the standard calcium solution.

Triethanolamine, 50 per cent aqueous solution. Mix equal volumes of triethanolamine and distilled water.

Methyl red indicator. 0.1 per cent. Dissolve 0.1 grams of methyl red in 100 ml of 50 per cent ethyl alcohol.

Procedure

Standardization of EDTA solution. Dilute a 5 ml aliquot of the standard calcium solution to about 50 ml, add 2 ml of the 50 per cent triethanolamine solution and stir. Add 3 ml of sodium hydroxide-sodium cyanide solution and 1 drop of Calcein indicator. Titrate under diffuse light with the EDTA solution. The endpoint is taken as the change in color of the solution from green to light brown. Titrate blanks, with the same amounts of reagents but with distilled water substituted for the calcium standard.

$$\text{Normality of EDTA solution} = \frac{0.05}{\text{ml EDTA solution used, corrected for blank titration}}$$

Determination of calcium in plant material. Ash an 0.3 to 1.0 gram sample of the plant material as described on page 34. Transfer an aliquot of the ash solution expected to contain from 0.4 to 2.0 mg of calcium to the titration vessel, add reagents and titrate as described for standardization of EDTA solution to a permanent brown endpoint. Sufficient excess of the EDTA solution (0.5 to 1.0 ml) should be added to insure that all calcium phosphate has dissolved as noted by persistence of the brown endpoint after stirring for one minute. Back titrate the excess EDTA with standard calcium solution to the green color of the indicator. Titrate with EDTA to the exact brown endpoint. Record total EDTA volume used, volume of standard calcium solution used, and volume of EDTA used for blank determination.

Calculation

Milliequivalents calcium = (total ml EDTA − ml EDTA for blank) × N of EDTA − (volume standard calcium solution × N of standard calcium solution)

Since the equivalent weight of calcium in this titration is 20.00, the per cent calcium in the sample is obtained by the following calculation:

$$\text{Per cent calcium} = \frac{\text{millequivalents calcium} \times 20.00}{\text{mg sample represented by aliquot taken}} \times 100$$

Determination of magnesium in plant material. In this determination the sum of magnesium and calcium is determined by titration with EDTA solution using Eriochrome Black T indicator. The amount of magnesium is then determined by the difference in this titration and the titration using Calcein indicator (calcium titration).

Transfer an aliquot of the plant ash solution containing from 0.02 to 0.10 milliequivalents of calcium plus magnesium

to a 100 ml beaker. Usually an aliquot representing 0.1 gram or less of dry plant sample is sufficient. Add 2 ml of 50 per cent triethanolamine solution, stir, add 3 ml of the ammonium chloride-ammonium hydroxide solution, 6 drops of methyl red solution, and 5 drops of Eriochrome Black T indicator. The methyl red indicator is added to provide a yellow background so that the final indicator color change is from red to green rather than the usual red to blue color. Most analysts find the red to green transition easier to observe than the red to blue. Titrate the solution with the standard EDTA solution until the green color persists, add 1.0 ml excess EDTA solution to insure that all calcium and magnesium salts are dissolved as determined by persistence of the green color when the solution is stirred for an additional minute. Back titrate the solution with standard calcium solution to the red endpoint and then approach the green endpoint by dropwise titration with the EDTA solution. Record separately the total volume of EDTA and standard calcium solution used. Run a blank determination using distilled water instead of sample but with addition of all other reagents.

Calculations

(1) Milliequivalents of calcium plus magnesium in the aliquot =

$$(V\ EDTA \times N\ EDTA - V\ std\ Ca \times N\ std\ Ca)_{Sample} - (V\ EDTA \times N\ EDTA - V\ std\ Ca \times N\ std\ Ca)_{Blank}$$

: Where

V EDTA = total volume EDTA solution used in titration
N EDTA = normality of EDTA solution
V std Ca = volume of standard calcium solution used in back titration
N std Ca = normality of standard calcium

(2) Milliequivalents of magnesium are obtained by subtracting from the value obtained for milliequivalents of calcium plus magnesium (equation 1 above) the value obtained for milliequivalents of calcium *in the same size aliquot* in the titration for calcium alone.

(3) The equivalent weight of magnesium in this titration is 12.16, therefore: Per cent of magnesium in the sample =

$$\frac{\text{milliequivalents magnesium} \times 12.16}{\text{milligrams sample represented by sample taken}} \times 100$$

Comments

Lighting conditions for the titrations are critical. The endpoint in the calcium titration using the Calcein indicator is most distinct under subdued light. The calcium plus magnesium titration using the Eriochrome Black T indicator requires rather strong transverse illumination. An ordinary desk lamp arranged so that the light shines through the solution toward the operator has been found satisfactory.

Glassware used in the determinations should be washed with dilute (approx. 1–3 N) hydrochloric acid to remove traces of calcium and magnesium that may have deposited as soap films in washing, and then thoroughly rinsed with distilled water.

There are several possible interferences in the determinations. Iron, manganese, zinc, and copper may interfere by reacting with the indicators thus obscuring endpoint detection in the titration. Zinc and copper, in amounts encountered in plant materials, are effectively masked

by the cyanide so that there is never any interference from these elements. Iron and manganese interference is prevented over a limited range by the addition of triethanolamine. The amounts of iron and manganese that may be tolerated in the aliquot taken for titration are (1) in the calcium titration, 2 mg of iron and 0.3 mg of manganese and (2) in the calcium plus magnesium titration, 0.2 mg iron and 0.015 mg of manganese. If aliquots of the plant ash solution representing 0.1 gm or less of the dry plant material are taken it is unlikely that interferences from either iron or manganese will be observed in either the calcium or the calcium plus magnesium titrations.

Visual evidence of interference from excess iron in the calcium titration is the appearance of a deep yellow color when the triethanolamine and sodium hydroxide-sodium cyanide solution is added. Interfering amounts of manganese causes a green coloration in the solution when the triethanolamine and alkali are added. These off colors prevent the detection of the Calcein endpoint and smaller aliquots should be taken for the titration. In the calcium plus magnesium titration, amounts of iron and manganese that are too small to cause the initial off colors may, however, react with the Eriochrome Black T indicator to cause an irreversible pink-yellow color change at the endpoint rather than the reversible red to green observed in successful titrations. If the pink-yellow indicator color is observed the sample should be discarded and a smaller aliquot taken.

Phosphorus always present in plant samples, precipitates calcium and magnesium at the alkaline reaction of the solution. However, addition of a small but definite excess of the EDTA solution and stirring for a minute or two will release all the calcium and magnesium to the EDTA. The excess EDTA is then back titrated with standard calcium solution and the true endpoint approached by slow titration with EDTA.

Chloride by the Mohr Method

Chloride concentrations in plant material range from 0.003 to 10 per cent of dry matter.

The chloride concentration in plant material may be estimated with sufficient sensitivity to determine whether silver sulfate or water only is required for nitrate extraction (page 44) by titrating an aliquot of the water extract with silver nitrate, using potassium chromate as an indicator to establish the end point. For more sensitive and accurate determinations of chloride, the potentiometric procedure on page 39 is recommended.

Reagents

Potassium chromate indicator. Dissolve 5 gm of potassium chromate (K_2CrO_4) in water, add silver nitrate until a slight permanent red precipitate is produced, filter and dilute to 100 ml.

Standard silver nitrate solution. Dissolve 4.791 gm of silver nitrate ($AgNO_3$) in water and dilute to 1 liter; 1 ml of this solution is equivalent to 1 mg of chloride. Check the silver nitrate solution by titration against a standard solution of sodium chloride.

Procedure

Weigh 100 mg samples of ground plant material into centrifuge tubes. Add 25 ml of distilled water and stopper tubes with rubber stoppers. Place on shaker for 10 minutes and then centrifuge for 10 minutes at 4000 rpm. Take a 20 ml aliquot of this extract and pipette it into a white porcelain dish. Add 1 ml of potassium chromate indicator. Titrate with silver nitrate to a light brown color; the light brown color is due to the formation of silver chromate when all of the chloride has been precipitated. Titration under a yellow light aids in defining the end point more sharply. The blank to be subtracted from this titration is obtained by substituting distilled water for the extract and titrating to the same end point.

Calculations

$$\text{Per cent Cl} = \frac{\text{Vol. } AgNO_3 \text{ (ml)}}{\text{wt. sample (mg)}} \times 100$$

(1 ml of $AgNO_3$ is equivalent to 1 mg Cl.)

Chloride by Potentiometric Titration

The method of Kolthoff and Kuroda (1951) has been adapted *(Johnson, et al,* 1958) for the determination of chloride in plant materials. The method, which can determine amounts of chloride as low as 35 ppm with a precision of 5 per cent, requires the use of silver-silver chloride electrodes and a potentiometer or pH meter. Except for the special equipment required the method is as simple as the much less sensitive and less precise Mohr method.

The method is based on the observation of the potential (pH meter reading if a pH meter is used) developed by a silver-silver chloride electrode system immersed in a "supporting solution" containing suspended silver chloride. The endpoint in an unknown titration is obtained by titrating the unknown in the same concentration of "supporting electrolyte" to the same apparent equivalence potential with dilute silver nitrate.

Equipment

Student-type potentiometer or pH meter.

Silver-silver chloride electrodes. Polish a piece of 16 gauge silver wire, about 15 cm long, with fine sandpaper or emery cloth and clean by successive immersions in alcohol, ammonia and 3 N nitric acid. Rinse between each treatment with distilled water. Immerse 5 cm of the silver wire in 0.1 N hydrochloric acid, connect the silver wire through a series resistance to the positive pole of a 2 volt dry cell. Immerse a platinum wire in the same solution and connect to the negative pole. Adjust the resistance so that a current of 3 to 4 milliamperes flows through the circuit. After 15 to 30 minutes the wire should have a good deposit of brown silver chloride. Remove the wire and rinse with distilled water. Store the electrode in distilled water when not in use. The electrode should be aged a few days before use but then should be useful for several months.

Reference electrode. A convenient reference electrode for this titration is illustrated in Fig. 1. Seal a short piece of platinum wire (28 gauge) in the end of a piece of 3 to 5 mm Pyrex glass tubing. Add about 0.1 gm of quinhydrone to the tube and then add buffer solution, (composed of 1.02 gm of potassium acid phthalate, 14.8 ml of 0.1 N sulfuric acid and diluted to 100 ml with distilled water), using a piece of platinum to dislodge air bubbles that are entrapped. Seal the platinum wire in position in the glass tube with De Khotinsky cement. Provide suitable mechanical and electrical connections from the electrodes to the potentiometer or pH meter.

Salt Bridge. Prepare from 8 mm Pyrex glass tubing the salt bridge shown in detail in Fig. 1. Note that the small asbestos fiber, which is fitted into the drawn out tip of the bridge to provide electrical contact, should be sealed in sufficiently tightly to prevent excessive leakage of the saturated potassium nitrate solution. The offset design of the silver-silver chloride electrode and the salt bridge permits the insertion of the electrode, salt bridge, buret tip and stirrer in a small beaker. Prepare chloride-free potassium nitrate by recrystallizing three times. Add solid potassium nitrate to the bridge and then pour in sufficient saturated potassium nitrate solution to cover all the solid potassium nitrate from the asbestos tip to an inch above the solid. Replace saturated potassium nitrate solution and solid potassium nitrate as necessary to make up for that lost through the asbestos wick.

Semi-micro or micro burets.

Electric stirrer, variable speed, motor, propellor or screw-type glass stirrer.

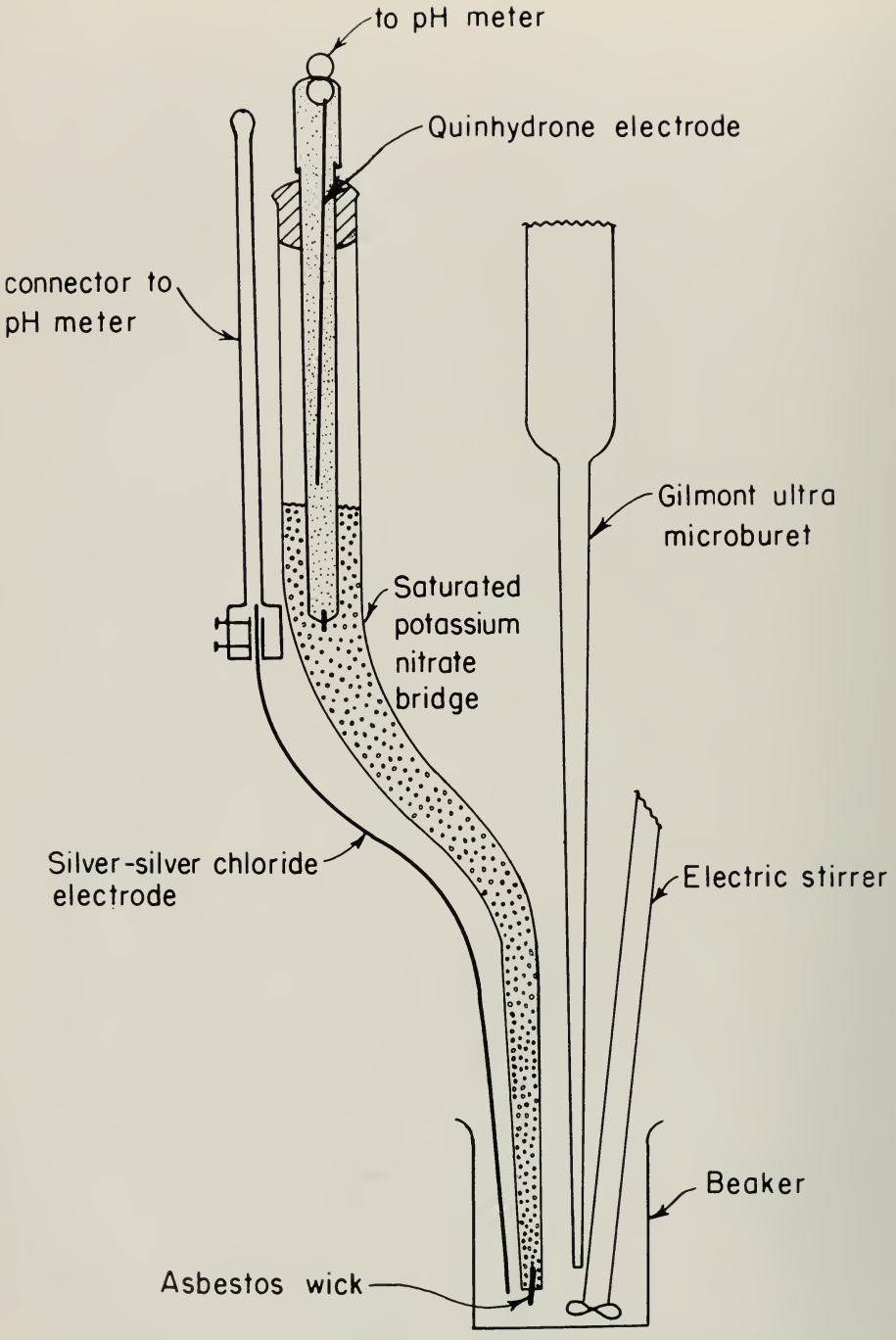

Fig. 1. Titration Assembly for Potentiometric Determination of Chloride.

Reagents

Silver chloride suspension. Precipitate silver chloride by mixing 0.1 N sodium chloride and 0.1 N silver nitrate, 500 ml of each solution will provide sufficient silver chloride for many determinations. A slight excess of either chloride or silver should be used to give a good precipitate. Wash the precipitate thoroughly with distilled water and transfer to a brown bottle. Replace the supernatant daily for about 10 days with distilled water to remove traces of excess of either silver or chloride ion. Make the volume of the suspension to 1 liter.

Silver nitrate, 0.1 N. Dissolve 8.5 grams of silver nitrate in 500 ml distilled water. Store in dark bottle.

Silver nitrate 0.01 N. Dilute 10.00 ml of the 0.1 N silver nitrate to 100 ml with distilled water. Check the normality of the dilute silver nitrate by titration of standard potassium chloride solution before use. The dilute silver nitrate should be prepared at frequent intervals and stored in a dark bottle.

Standard Potassium Chloride Solution. Dissolve 0.746 grams of pure potassium chloride (KCl) in distilled water and dilute to 1 liter in a volumetric flask. This solution is 0.01 N with respect to chloride and is to be used in standardizing the silver nitrate solution for each day's determination.

Supporting Electrolyte. Dissolve 101 grams analytical grade potassium nitrate in water, add 62 ml concentrated nitric acid and make to 1 liter. If blanks appear to be high, it may be necessary to remove chloride from the potassium nitrate by recrystallization and from nitric acid by distillation.

Procedure

Transfer 1.00 gram (or less if expected chloride content is high) of the plant material to a 100 ml beaker, add 10 ml distilled water, cover with watchglass and boil gently for about 10 minutes. Filter the suspension on a sintered glass funnel, washing with several small portions of hot distilled water. Total volume of filtrate and washings should be about 30 ml if the titration is to be done on the whole solution. Alternatively the filtrate and washing may be made to a standard volume and aliquots taken for the titration.

Separation of the solution and the solid residue by centrifuging is often preferable to filtration on sintered glass funnels because of the tendency of the funnels to become clogged with plant residue. Direct titration of the suspension, although recommended by some, tends to give slightly high results with a drifting end point. This is especially serious when the chloride concentration of the plant sample is very low.

Transfer the solution to a 50 ml beaker, add sufficient 1 N nitric acid to make the solution approximately 0.1 N with respect to nitric acid. At the same time prepare the reference solution, consisting of 25 ml distilled water, 2.5 ml of the supporting electrolyte and 2 drops of the silver chloride suspension. Immerse the electrode system in the reference solution, start gentle stirring, close the circuit to the potentiometer or pH meter and observe the "apparent equivalence potential" or the reading on the pH meter. Remove the reference solution and put the sample solution in place. Lock the push button in the closed position and titrate, with gentle stirring, with the 0.01 N silver nitrate until the indicated potential or pH meter reading is the same as that given by the reference solution. Record the volume of the silver nitrate solution required. One milliliter of 0.01 silver nitrate is equivalent to 0.355 mg of chloride (Cl). For a 1 gram sample: ml 0.01 N silver nitrate $\times$ 355 = ppm chloride.

Magnesium by the Thiazole Yellow Method

Magnesium concentration in plant material may range from 0.02 to 2.50 per cent of dry matter.

Magnesium is determined colorimet-

rically by the thiazole yellow method as described by Drosdoff and Nearpass (1948). The procedure developed by these workers uses a compensating solution to eliminate some of the interferences that have prevented the effective use of this reagent in the determination of magnesium. Calcium, aluminum, manganese and phosphate have an effect on the intensity of the color of the magnesium-thiazole yellow lake, therefore a compensating solution which contains these ions in amounts much larger than found in the ash sample is added to blanks, standards, and samples to minimize the variations in the content of these ions in the plant sample. A starch solution is added to stabilize the suspension of the magnesium-thiazole yellow lake. The useful range of the method is rather limited, in the order of 2 to 150 micrograms of magnesium in the aliquot, but this objection is not serious because the size of the aliquot can be adjusted to fall within the range. The standard deviation of a single determination is $\pm$ 0.028 or about 6.0 per cent of the mean for a sample containing 0.47 per cent magnesium (dry basis).

Equipment

Muffle furnace.

Spectrophotometer or photo-electric colorimeter with filter transmitting at a maximum near 525 mμ.

Reagents

Thiazole yellow. 0.10 per cent solution in water. Dissolve 0.10 grams of thiazole yellow in 100 ml distilled water and store in dark bottle. Titan yellow appears to be the same or similar to thiazole yellow and may be satisfactory. Different lots of the dye appear to behave differently and it may be necessary to check on the performance of several lots.

Hydroxylamine hydrochloride, 5 per cent. Dissolve 25 grams of hydroxylamine hydrochloride (H_2NOH HCl) in 500 ml of distilled water.

Sodium hydroxide, 2.5 N. Dissolve 100 grams of sodium hydroxide in 1 liter of distilled water.

Starch solution, 2 per cent. Add distilled water to 2 grams of soluble starch to make a thin paste. Pour this paste into 100 ml of boiling water with stirring. Cool, filter if turbid. This solution should be prepared daily as needed.

Compensating solution. Dissolve 3.7 grams of calcium chloride [$CaCl_2 2H_2O$], 0.74 grams of aluminum sulfate [$Al_2(SO_4)_3 \cdot 18H_2O$], 0.36 grams of manganous chloride [$MnCl_2 4H_2O$], and 0.60 grams of sodium phosphate [Na_3PO_4] in 1 liter of distilled water containing 10 ml of concentrated hydrochloric acid.

Starch compensating solution. Mix equal volumes of the freshly prepared starch solution and the compensating solution.

Magnesium standard solutions. Dissolve 250 mg of reagent grade magnesium metal in dilute hydrochloric acid solution (150 ml of water plus 10 ml of concentrated hydrochloric acid). Transfer to 250 ml volumetric flask, make to volume and mix. One ml of this solution contains 1 mg of magnesium (Mg). Dilute 25 ml of this solution to 1 liter in a volumetric flask to provide a standard solution containing 25 micrograms of magnesium per ml.

Alcoholic sulfuric acid. Add 50 ml concentrated sulfuric acid to 950 ml of 95 per cent ethanol.

Procedure

Transfer the sample, 0.1 to 1.0 grams, to a Coors porcelain crucible (No. 000), moisten with 2 ml or more of the alcoholic sulfuric acid. Burn off excess alcohol and ash in the muffle furnace at 600°C for four hours or overnight if convenient. The ash should be free of carbon. Moisten the ash with distilled water and a slight excess of dilute hydrochloric acid. Warm slightly to insure solution of ash material. Transfer the ash solution to

a 25 ml volumetric flask with thorough washing. Make to volume and mix. Transfer an aliquot to contain from 25 to 100 micrograms of magnesium to a 50 ml volumetric flask. The exact amount to be taken will depend on the useful range of magnesium to be determined with the particular colorimeter used.

Add, in order and with mixing between additions, 1 ml of the hydroxylamine solution, 5 ml of the starch compensating solution and exactly 1 ml of the thiazole solution, and finally, after thorough mixing, 5 ml of the 2.5 N sodium hydroxide solution. Bring to 50 ml volume, mix and allow to stand from 10 to 30 minutes before reading the color intensity at 525 mμ. Blanks and standards are run in exactly the same manner. The amount of magnesium is obtained from the standard curve and the concentration of magnesium calculated, taking into consideration the amount of dry material represented by the aliquot used for color development.

Nitrate by the Diphenylamine Test

A preliminary test of the ground plant material for nitrate with diphenylamine reagent has been found to be an expedient way of detecting those samples free or nearly free of nitrate. By eliminating these samples for quantitative analysis, a considerable saving of time is effected even though 90 per cent of the samples remain to be run by the longer phenoldisulfonic acid method.

Equipment

Porcelain spot plates provided with 12 depressions each, one small spatula and two glass bottles fitted with ground glass eye droppers for dispensing distilled water and the diphenylamine reagent.

Reagents

Distilled water.

Diphenylamine, 0.2 gm in 100 ml of nitrate-free concentrated sulfuric acid.

Procedure

To each of the depressions in one of the spot plates, add 1 drop of distilled water. Add to the water, by means of the spatula, 2 to 3 cubic millimeters of ground plant material. Repeat this procedure in rotation for other samples for each of the remaining depressions. Rotate the spot plate in a horizontal plane so as to mix the plant material with the water. Add in rapid succession 5 drops of diphenylamine reagent to the depression so that each drop falls in a different position on the sample. Do not stir or mix the materials. Observe the formation of the blue color which develops within a few minutes.

Diphenylamine reagent for 4 to 12 utes. Diphenylamine reagent for 4 to 12 samples may be added before readings of color formation are recorded. From 60 to 100 samples can be observed by this test in one hour.

Interpretation

A blue to blue-black color develops instantly for samples with nitrate-nitrogen concentrations above 1,000 ppm. The color produced becomes less intense and develops more slowly as the nitrate concentration decreases. For plant material contaning around 100 ppm nitrate-nitrogen several minutes are required for formation of the blue color, which at times appears as pinpoints about isolated particles of plant material. From the diagnostic point of view these samples indicate a nitrogen deficiency and may therefore be eliminated from further analytical consideration. In samples low in nitrate, a brown coloration due to carmelization of organic matter by the sulfuric acid gradually develops; in time, this obscures the faint blue color of the test. In leaf blade material from grasses or cereals, chlorophyll tends to mask the blue color of samples that are low in nitrate, but even here you will be able to detect traces of blue color after a little experience.

Nitrate Nitrogen in the Presence of Excess Chloride

Nitrate nitrogen concentrations in plant samples range from 0 to 35,000 ppm of dry matter.

The nitrate concentration of water extracts of plant material may be estimated accurately with phenoldisulfonic acid when chloride in excess of 1 per cent of the dry plant material is removed with silver sulfate and when the organic matter of the extract is destroyed with hydrogen peroxide (Johnson and Ulrich, 1950). The silver sulfate in the extract precipitates the chloride ions and almost always results in a surplus of silver ions. If soluble silver is not removed prior to the digestion step, the silver ions decompose the hydrogen peroxide before the organic matter is oxidized completely. The excess silver is removed with sodium phosphate to prevent decomposition of the hydrogen peroxide but a turbidity of calcium and iron phosphate appears when the ammonium hydroxide is added for color development. This difficulty can be overcome by adding a sequestering or chelating agent, as for example ethylenediaminetetraacetate (EDTA), before adding the ammonium hydroxide.

With these modifications of the phenoldisulfonic acid procedure, 30 to 50 nitrate determinations can be run conveniently in an 8-hour day when starting from the ground plant material. The accuracy of a single determination (standard deviation) is $\pm$ 6.6 per cent from day to day and $\pm$ 2.5 per cent within a day for readings of 100 to 350 on a colorimeter such as the Klett-Summerson. The method as outlined has a range of 100 to 3,500 ppm of nitrate nitrogen in the dry plant material when the colored solution is diluted to 100 ml. More deeply colored solutions may be diluted to a larger volume before reading on the colorimeter. Make certain that excess ammonia is present after diluting.

Equipment

In addition to the usual laboratory equipment, the analytical work will be facilitated by use of a photoelectric colorimeter, a 25 ml automatic pipette for dispensing the silver sulfate solution, and a machine for shaking the samples, and a centrifuge.

Reagents

Silver sulfate solution. Dissolve 3.5 gm of silver sulfate (Ag_2SO_4) in 1 liter of distilled water. Boiling speeds up the rate of solution of the silver sulfate.

Sodium phosphate reagent. Dissolve 138 gm sodium dihydrogen phosphate monohydrate ($NaH_2PO_4H_2O$) in 800 ml of water. Adjust the pH of the solution to 6.5 with 5N sodium hydroxide. Make to 1 liter.

Calcium carbonate suspension. Suspend 1 gm calcium carbonate (nitrate-free) in 200 ml of distilled water.

Hydrogen peroxide, 30 per cent, containing less than 9 ppm of nitrate nitrogen. Hydrogen peroxide supplied by the Buffalo Electro Chemical Co. and by Merck and Company (microchemical grade), has been found satisfactory.

Phenoldisulfonic acid. Dissolve 25 gm of pure white phenol in 150 ml of concentrated sulfuric acid, add 75 ml of fuming sulfuric acid (13–15 per cent sulfur trioxide) and heat over a steam bath at 95° to 100°C for 2 hours or more. Store in a tightly stoppered brown bottle.

Sequestrene or Versene solution, 20 per cent. Stir 40 gm ethylenediaminetetraacetic acid dihydrate (EDTA—acid form) in 100 ml water. Add 1:1 ammonium hydroxide until dissolved. Filter if necessary. Make to 200 ml.

Dilute sequestering solution. Dilute 10 ml of the 20 per cent EDTA solution to 2 liters.

Ammonium hydroxide 1:1. Add concentrated ammonium hydroxide to an equal volume of distilled water.

Standard nitrate solutions. Dissolve 7.22 gm of pure potassium nitrate

anhydrous (KNO_3) in water and make up to 1 liter. Then dilute 5 ml of this solution to 1 liter. The latter solution has a concentration of 5 ppm of nitrogen or 1 ml = 5 micrograms of nitrogen. (0.005 mg.)

Procedure

Weigh 100 mg ground plant material into centrifuge tubes. Add 25 ml silver sulfate solution and 1 ml sodium phosphate solution. Stopper and shake for 10 minutes. Centrifuge at 4000 rpm for 10 minutes, or filter through dry No. 12 Whatman folded filter paper (do not wash residue). Transfer aliquots of 10 ml (containing not more than 0.15 mg nitrate nitrogen) to 100 ml beakers. Avoid carrying over solid particles when aliquoting. Add 2 ml of the calcium carbonate suspension and 1.0 ml 30 per cent hydrogen peroxide. Cover with watchglass and digest on the steam bath for 2 hours. Do not substitute a sandbath for a steambath. Samples dehydrated on a sandbath become overheated and a loss of nitrate results. Timing here is critical. Uncover, take to dryness and leave on the steam bath for an additional half hour to destroy any residual hydrogen peroxide.

Deflagration of the residue after digestion and dehydration of the extract on the steam bath indicates incomplete oxidation of the organic matter and the presence of hydrogen peroxide. The difficulty may be corrected by increasing the time of digestion or by raising the temperature of the solutions while they are digesting. Remove from steam bath, cool, and add 2 ml phenoldisulfonic acid. This reagent should be added rapidly, covering the entire residue at once. Break up any crust or solid particles with a stirring rod. After 10 minutes, add approximately 70–75 ml of dilute sequestering solution. Add an excess of 1:1 ammonium hydroxide (about 15 ml). Ascertain excess ammonium hydroxide by odor or test paper. Cool and make to 100 ml volume. The color of the solution after adding excess ammonium hydroxide should be a pure yellow. A brown tinge in the solution is an indication of incomplete oxidation of organic matter in the peroxide digestion step. The determination should be repeated using more (2 to 4 ml as determined by trial and error) hydrogen peroxide. Measure intensity with a photoelectric colorimeter, using a 2 cm absorption cell and blue filter. A 48 microgram standard is prepared with 25 ml of a 5 ppm nitrate-nitrogen standard solution plus 1 ml sodium phosphate solution and aliquoting 10 ml as with the extracts.

Calculations

$$\text{ppm } NO_3^- - N = \frac{\text{R-B} \times \text{microgram N in std.}}{\text{Rstd}} \times \frac{26 \text{ ml}}{\text{vol of aliquot}} \times \frac{1000}{\text{wt. of sample in mg.}}$$

Where R = reading of sample

B = reading of blank

Rstd = reading of a standard

Nitrate Nitrogen in the Absence of Excess Chloride

When the chloride concentration of the plant sample is less than 1 per cent on the dry basis, use of silver sulfate, sodium phosphate, and sequestering solution may be omitted from the procedure. The sample is extracted with distilled water, thereafter all other steps in the procedure are the same as for the determination of nitrate in the presence of a high concentration of chloride.

Potassium by the Cobaltinitrate Method

Potassium concentrations in plant materials range from 0.2 to 11 per cent of dry matter.

The precipitation of potassium as potassium cobaltinitrite and the titration of

the precipitate with potassium permanganate has been found to be a convenient method of determining the potassium concentration of plant material when sodium is not to be estimated simultaneously (Hibbard and Stout, 1937). By following this procedure, an operator can, on the average, analyze 30 to 50 samples a day, starting with the ground plant material. The standard deviation for a single determination is $\pm$ 0.062, where the sample contains 2.53 per cent potassium. This is an error of $\pm$ 2.4 per cent for a single determination.

Equipment

Electric furnace, one 5-ml automatic pipette, and two 10 ml burettes calibrated in 0.05-ml intervals.

Reagents

Cobaltinitrite solution. Dissolve 113 gm of cobaltous acetate in 300 ml of hot water and 100 ml of glacial acetic acid.

Dissolve 200 gm of sodium nitrite in 400 ml of hot water.

Mix equal parts of cooled solutions, force air through the mixture until evolution of red fumes ceases, and protect from light and ammonia fumes. If the mixture is not quite clear, filter before using.

Sodium oxalate, 0.05 N. Dissolve 3.350 grams anhydrous sodium oxalate ($Na_2C_2O_4$) in water, add 10 ml of concentrated sulfuric acid and make to a volume of one liter.

Potassium permanganate, 0.05 N. This must be free of oxides of manganese. Weigh out on a watch glass approximately 3.2 gm of potassium permanganate ($KMnO_4$), dissolve in two liters of distilled water, boil 10 to 15 minutes, allow to cool over night and then filter through a sintered glass funnel. Protect this solution from dust and reducing fumes. Standardize this solution against the standard oxalate.

Sulfuric acid, 1:1.

Acetic acid, 2 per cent by volume. Dilute 20 ml of glacial acetic acid to one liter with distilled water.

Filter aid for filtering. Suspend 20 gm of talc powder or analytical grade Celite in water and by decanting the fine particles into another beaker, remove the coarse particles that settle out immediately. Have approximately 500 ml of water present with the filter aid, and then add carefully 250 ml of concentrated sulfuric acid. This will raise the temperature of the suspension and cause a frothing of the very fine particles. Discard the froth by decantation, and remove the oxidizable material that may be present in the remaining filter aid by heating the talc suspension with potassium permanganate in the presence of sulfuric acid. Wash the treated filter aid with water until all permanganate color is removed. Test the filter aid as a filter by adding enough suspension to a sintered glass funnel (coarse porosity) to form a uniform layer of filter aid 0.2 to 0.7 mm thick. This layer should be thick enough to prevent the precipitate from staining the sintered glass plate and yet permit rapid filtration and washing of the precipitate when a clean filter is used.

Alcohol-sulfuric acid solution: Add 50 ml of concentrated sulfuric acid to 950 ml of 95 per cent ethyl alcohol.

Standard potassium solution: Dissolve 0.763 gm of pure potassium chloride (KCl) in water and make to 1 liter. Five ml of this solution contain 2.0 mg of potassium (K).

Procedure

Place 100 mg of ground plant material into a small porcelain evaporating dish (Coors 000). Add 1 ml of alcoholic sulfuric acid and set afire, using this reagent in a pipette as a torch. Heat in an electric muffle at 550 to 600° C for three hours. Cool and add directly to the evaporating dish 5 ml of 2 per cent acetic acid and 5 ml of the cobaltinitrite solution. Let stand overnight. Filter on a sintered glass

funnel with a mat of filter aid 0.2 to 0.7 mm thick. Wash the precipitate three times with small amounts of distilled water. Transfer the precipitate and filter aid with water to a 100 ml beaker. Add 2 ml of 1:1 sulfuric acid and a small amount of 0.05 N potassium permanganate. Heat on a hot plate until the color of the permanganate solution almost disappears. Add more permanganate until a pink color persists upon boiling on the hot plate. Add sodium oxalate until the solution is colorless, recording the volume of oxalate, and then back titrate with permanganate to a very pale pink color. With every set of determinations include for analysis three reference plant samples of known potassium concentration and three standard potassium chloride solutions containing 2 mg potassium each. Dehydrate the standard solutions on the steam bath (but do not heat in muffle) prior to adding the precipitating reagents and use the results for calculating the potassium equivalence of the permanganate titrations of the unknowns.

Calculations

$$\text{Per cent potassium} = \frac{V \times \text{mg Kstd}}{\text{Vstd}} \times \frac{100}{\text{wt. of sample (mg)}}$$

Where V = net volume permanganate for sample

Vstd = net volume permanganate for standard

Net volume, permanganate = volume added to give a pink color minus volume of oxalate used to decolorize permanganate

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Comments

When titrating the potassium cobaltinitrite precipitate, maintain a slight excess of permanganate reagent as long as any yellow precipitate remains. Do not allow any ammonia fumes to come in contact with the samples after they have been ashed in the muffle or during the storage period prior to filtration, otherwise high values will be obtained. Low values are obtained whenever the quantity of potassium in the sample exceeds 2.5 mg. A uniform temperature in the laboratory appears conducive to comparable results from day to day.

Potassium and Sodium by Flame Photometer

Potassium concentrations range from 0.2 to 11 per cent, and sodium concentrations from 0 to 10 per cent of dry matter.

The determination of potassium and sodium by a flame photometer is now a standard procedure for many laboratories. The details for their operation have been described by the manufacturers of these instruments and will not be reviewed here. Only the steps leading to the preparation of the sample for analysis and the calculation of the results will be considered here in detail. Ordinarily an operator can determine potassium and sodium on 30 to 50 samples per day with a precision of $\pm$ 2.5 per cent for potassium and $\pm$ 9.4 per cent for sodium. The latter figures are the standard deviations expressed in per cent of the mean (coefficients of variability) for samples with an average concentration of 2.53 per cent potassium and of 0.48 per cent sodium.

Equipment

Flame photometer.

Metal trays. Aluminum cake pans, 18″ × 12″ × 2″ have been found useful.

Table 1. Preparation of Standard Potassium Plus Sodium Solutions.

Solution to contain identical potassium and sodium concentrations	Take ml of		Take ml of 37.5 ppm Li Soln.*	Dilute to ml
	100 ppm K Soln.	100 ppm Na Soln.		
ppm.				
2	2	2	10	100
4	4	4	10	100
6	6	6	10	100
8	8	8	10	100
10	10	10	10	100
15	15	15	10	100
20	20	20	10	100
30	30	30	10	100
40	40	40	10	100
	1000 ppm K Soln., 1000 ppm Na Soln.			
50	5	5	10	100
60	6	6	10	100
70	7	7	10	100
80	8	8	10	100

Avoid contact of fingers with the solutions because of sodium contamination from perspiration. Store the solutions in polyethylene bottles to avoid solution of sodium from glassware.
* If a direct reading flame photometer is used, omit the addition of lithium solution.

Reagents

Note: Lithium solutions are prepared only for use with internal standard flame photometers as for example, the Perkin-Elmer instrument.

375 ppm lithium standard. Dissolve 37.25 gm of anhydrous lithium nitrate ($LiNO_3$) in distilled water and make to 10 liters.

37.5 ppm lithium nitrate in nitric acid solution. Dilute 1,000 ml of reagent (previous paragraph) and 1,000 ml concentrated nitric acid with distilled water to 10 liters. Each time a new 10-liter batch of lithium nitrate in nitric acid is prepared, prepare a new set of standard solutions. This will insure that the concentration of lithium in the standards and samples will be identical (37.5 ppm) whenever a run is made.

Potassium standard, 1,000 ppm potassium (K). Dissolve 1.907 gm of reagent grade potassium chloride (KCl) in distilled water and make to one liter. Dilute 100 ml of the 1,000 ppm potassium (K) solution to one liter with distilled water. This solution contains 100 ppm potassium (K).

Sodium standard, 1,000 ppm sodium (Na). Dissolve 2.542 gm sodium chloride in distilled water and dilute to one liter. Dilute 100 ml of the 1,000 ppm sodium solution to one liter with distilled water. This solution contains 100 ppm sodium (Na).

Prepare dilute standards containing sodium and potassium as indicated in table 1.

Alcoholic Sulfuric Acid. Add 50 ml concentrated sulfuric acid to 950 ml 95 per cent ethyl alcohol.

Procedure

Weigh 50 or 100 mg of the dry ground plant material into 35 ml capacity (Coors No. 000) porcelain evaporating dishes. Moisten with 1 or 2 ml of alcoholic sulfuric acid. Ignite, using the reagent in a pipette as a torch. Ash the samples in the muffle at 550–600°C for 3½ hours.

When cool, place the dishes in a metal tray and add 10 ml of the 37.5 ppm lithium nitrate in nitric acid to each dish with an automatic pipette.

Next add a little distilled water to the metal tray and place the tray on a hot plate. Heat the tray until the ash is in solution except for traces of silica and carbon; it is not necessary for the water to boil. Remove the tray from the hot plate and allow to cool.

Filter the samples through No. 12 folded Whatman filter paper into 100 ml volumetric flasks. Wash the dishes and filter paper carefully with distilled water. This will take approximately 50 ml of water. Bring the samples to volume and mix. Read the samples on the flame photometer for potassium and sodium content. Potassium is determined at 766.5 mμ and sodium at 589.3 mμ.

If a direct reading flame photometer is used, procedure is similar except that lithium nitrate is omitted from the nitric acid used for solution of the ash. Likewise lithium nitrate is omitted from the standards. Standards are run at appropriate ranges to ensure that a valid relationship between transmission reading and sodium or potassium content of the ash solution is obtained.

It should be appreciated that for most exact work the composition of the standards should be near that of the solution which is being analyzed. However, except in certain research analyses, it is usually not necessary to spend time preparing these special standards.

Calculations

Prepare a calibration curve by plotting per cent transmission against potassium or sodium concentration in ppm. The range chosen will depend on the amount of potassium or sodium in the samples. From the calibration curve, read the concentration of potassium or sodium in the sample as ppm in the solution. Calculate potassium or sodium as percent of dry sample from the following equation:

$$\text{Per cent K or Na} = \frac{\text{ppm K or Na in solution} \times 10}{\text{weight of sample (mg)}}$$

Phosphorus—acid soluble

Phosphate phosphorus concentrations range from 100 to 10,000 ppm of dry matter.

The phosphate in plant material which is soluble in 2 per cent acetic acid can be determined readily by developing the blue phosphomolybdate color under standard conditions and reading its intensity by means of a photoelectric colorimeter. Starting with the ground plant material, 30 to 60 samples can be analyzed by an operator during the course of the day with an error for a single determination (standard deviation) of less than ± 3 per cent for concentrations of 3,000 ppm phosphate-phosphorus in the dry material. The method as outlined may be applied without modification to dry plant material containing 200 to 4,000 ppm of phosphate phosphorus.

Equipment

Aside from the usual laboratory equipment it will be necessary to have a photoelectric colorimeter, a 25 ml automatic pipette and a dispenser for stannous chloride solution (figure 2).

Reagents

Ammonium molybdate in sulfuric acid (Reagent A). The final concentration of acid and molybdate in this reagent is important. Therefore, the following procedure has been adopted for the preparation of 2 liters of the reagent.

I. Preparation of approximately 14 *N* sulfuric acid. Add slowly with stirring, 580 ml of concentrated sulfuric acid

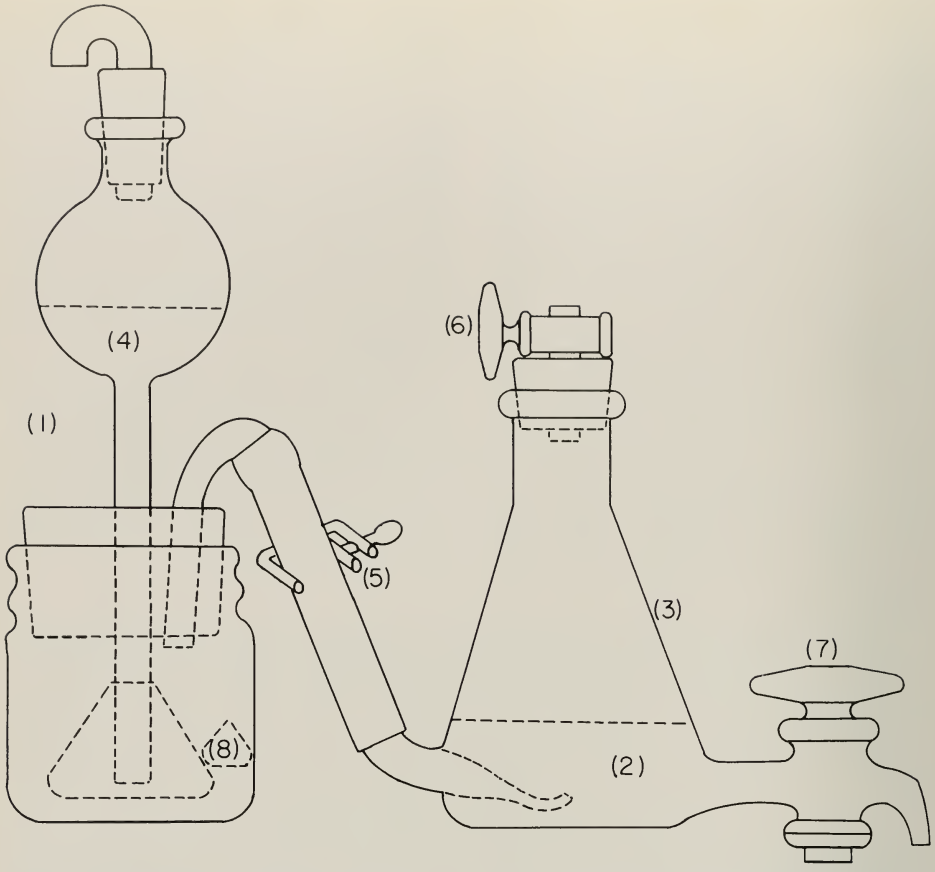


Fig. 2. Stannous chloride dispenser. The apparatus is essentially a Kipp generator (1) that is attached to an Erlenmeyer flask (3) containing the stannous chloride reagent (2) which may be added dropwise to the test solution by turning stopcock (7). To operate the generator, use marble pieces (8) at the bottom of (1) and 2.5 N hydrochloric acid in the funnel (4). Sweep out the air in the stannous chloride solution of flask (3) by opening the pinch clamp (5) and the stopcock (6). Allow the carbon dioxide to flow vigorously through the reagent for 5 to 10 minutes and then close stopcock (6). In an atmosphere of carbon dioxide, stannous chloride will keep satisfactorily for two to four weeks.

(arsenic free) to 920 ml of distilled water in a 3 liter beaker set in a pan of cold water. Cool. Determine the concentration of this acid by diluting a 10 ml aliquot to 100 ml. Titrate a 2 ml aliquot of this diluted acid with standard 0.1 *N* sodium hydroxide to the methyl orange or methyl red end point. From this titration calculate the volume of approximately 14 *N* sulfuric acid required to provide 2 liters of 10 *N* reagent.

Sample calculation: Assume that 28.0 ml of the 0.1 *N* sodium hydroxide were required for the titration of the 2 ml aliquot of the diluted sulfuric acid. From the relationship, volume of sulfuric acid × normality of sulfuric acid = volume sodium hydroxide × normality sodium hydroxide, the normality of the diluted sulfuric acid is:

$$\frac{28.00 \times 0.100}{2.00} \text{ or } 1.4 \text{ N.}$$

Thus, the more concentrated acid is 14 *N*

since it was diluted 10 fold for convenience in titrating with standard sodium hydroxide. Two liters of ammonium molybdate reagent in 10 *N* sulfuric acid will require M milliliters of the 14 *N* sulfuric acid, where $M = \frac{10 \times 2000}{14}$ or 1429 ml. The remaining 571 ml of solution for a final volume of 2000 ml is provided by the ammonium molybdate solution and distilled water, as given below.

II. Preparation of ammonium molybdate solution. Dissolve 50 grams of ammonium molybdate [$(NH_4)_6Mo_7O_{24}$-$4H_2O$] in 400 ml distilled water. Heat if necessary, but not above 60°C. Cool and then transfer to a 2 liter volumetric flask. Add the required amount of 14 *N* sulfuric acid and make to volume with distilled water when cool.

Stannous chloride solution (Reagent B). Transfer to a 150 ml beaker 1.0 gm granular tin metal, 20 ml of concentrated hydrochloric acid and 4 drops of a 4 per cent copper sulfate solution; dissolve the metal by heating on a steam bath. Dilute to 100 ml. This reagent will remain stable for 2 to 4 weeks when stored in an atmosphere of carbon dioxide, otherwise it must be prepared fresh daily. A convenient dispenser is illustrated in figure 2.

The stannous chloride (Reagent B) should be tested before each day's use in order to determine the number of drops to be added. Add the stannous chloride solution dropwise to a 2 ml aliquot of N/20 potassium permanganate until all color disappears. Use this same number of drops to develop the color in the samples.

Acetic acid 2 per cent by volume.

Hydrogen peroxide (phosphate free) 30 per cent.

Standard phosphate solution containing 0.005 mg phosphorus (P) per ml. Dissolve 2.196 gm. potassium dihydrogen phosphate (KH_2PO_4) in distilled water and dilute to 1 liter. Dilute 10 ml of this stock solution to 1 liter with 2 per cent acetic acid.

Procedure

Place 100 mg of ground plant material into a 50 ml centrifuge tube. Add 25 ml of 2 per cent acetic acid, stopped with a rubber stopper, and then shake for ten minutes. After shaking, place tubes in centrifuge for 10 minutes at 4000 rpm, or alternatively, filter through paper such as No. 12 folded or No. 30 Whatman. Pipette 2.0 ml (containing from 0.002 mg to 0.015 mg phosphorus, P) of the clear equilibrium extract into a 150-ml beaker. Add two drops of 30 per cent hydrogen peroxide. Hydrogen peroxide prevents formation of off colors which may occur with some samples. Place beakers on the steam bath and dehydrate; leave beakers on steam bath for at least ½ hour after dryness to remove all traces of hydrogen peroxide. Cool and then add 95 ml of distilled water. Next add 4 ml of reagent A to four beakers and then immediately add the appropriate amount of reagent B; repeat the operations for another set of four beakers. Read on photoelectric colorimeter between eight and twelve minutes after the addition of reagent B. With each set of samples, prepare three blanks, three controls and three standards. For the standards add 2 ml aliquots of a standard phosphate solution (1 ml equals 0.005 mg phosphorus, P) into 150 ml beakers and treat as described above. Similarly, add 2 ml of the acetic acid solution into 150 ml beakers for the blanks. Finally use the extract of a reference plant material for the controls. Read a control, blank and standard at the beginning, middle and end of each day's run.

With the Klett-Summerson photoelectric colorimeter use the red filter No. 66 (660 mμ) and the 4 cm light path of the 2 × 4 cm colorimeter cell. Set the instrument at zero with distilled water and make all readings with this setting. When

the instrument reads above 400 or less than 20, the determinations should be repeated using either smaller or larger aliquots of the extract as required.

If a colorimeter using a 1 cm cell is used, the amounts of phosphate to be taken may need to be increased accordingly. Alternatively, the total final volume of the blue solutions should be reduced to 25 ml, using one fourth the volume of reagents A and B.

Care in timing the readings after adding reagents A and B is important for accuracy. When correct amounts of stannous chloride solution are used, the color develops and then fades slowly for solutions with very low phosphate concentrations, whereas for solutions high in phosphate, but still within the range of the colorimeter, the color develops and fades rapidly. With experience, the intense colors can be read within 6 minutes and the faint colors as late as 10 to 12 minutes after addition of the reagents.

Calculations

$$\text{ppm } H_2PO_4^- - P = (R - B) \times \frac{\text{mg P std}}{\text{R std}} \times \frac{25 \text{ ml}}{\text{volume aliquot}} \times \frac{10^6}{\text{wt. of sample, (mg)}}$$

R = reading of sample

Rstd = reading of standard

mg Pstd = wt of P in standard = 0.01 mg P where a 2 ml aliquot is used in standard

B = reading of blank.

Phosphorus—Total

Total phosphorus concentrations in plant material range from 0.1 to 1.5 per cent of dry matter, with most frequent values in the range of 0.1 to 0.3 per cent.

The plant material is most conveniently ashed by the wet digestion method (page 32 ff) using a nitric acid predigestion and perchloric acid final digestion. Aliquots from the wet digestion for total sulfur may be used for total phosphorus. The determination of the phosphorus is made by the molybdenum blue method, using either stannous chloride or amidol as reducing agent.

Equipment

Wet digestion apparatus. Micro-Kjeldahl digestion rack. Micro-Kjeldahl flasks of 30 ml capacity.

Spectrophotometer or colorimeter. If a colorimeter is used, it must be provided with a blue filter transmitting in the region of 660 mμ.

Reagents

Concentrated nitric acid.

Reagents for development of molybdenum blue color by the stannous chloride method. See page 49 ff for description of these reagents.

Reagents for development of molybdenum blue color by the amidol method. Ammonium molybdate solution. 8.3 per cent in distilled water. Dissolve 8.3 grams of analytical grade ammonium molybdate [$(NH_4)_6Mo_7O_{24}$-$4H_2O$] in distilled water. Occasionally it may be necessary to add a small amount of ammonium hydroxide to facilitate solution. Make the solution to 100 ml and mix.

Perchloric acid. 60 per cent solution. If necessary prepare from 72 per cent solution by diluting 60 ml of 72 per cent to 72 ml.

Amidol reagent. Dissolve 2 grams of amidol, (2,4-diaminophenol dihydrochloride) and 40 grams of reagent grade

sodium bisulfite in distilled water and dilute to 200 ml. This reagent should be stored in a dark bottle and in a cool place. The amidol solution should be prepared fresh every ten days or when it begins to darken.

Standard phosphate solution. Stock solution containing 1 mg phosphorus per milliliter. Dissolve 1.099 grams analytical grade potassium acid phosphate (KH_2PO_4) (dried to constant weight at 105°C) in distilled water and make to volume in a 250 ml volumetric flask. Mold growth should be prevented by adding a small crystal of thymol or a few drops of chloroform to the solution.

Procedure

Ash 0.1 to 1.0 gram sample, depending on the expected phosphorus content and the particular method and colorimeter to be used. Place the plant material in a micro-Kjeldahl flask, add 5 ml concentrated nitric acid and 1 ml of perchloric acid for samples up to 0.5 grams. For samples from 0.5 to 1.0 grams use 10 ml concentrated nitric acid and 2 ml of perchloric acid. Allow the samples to digest overnight if practicable, if not, heat gently on the steam bath for one hour, taking care to avoid loss of sample by foaming. Transfer the flasks to a micro-Kjeldahl digestion rack and heat slowly to avoid excessive foaming. Increase the heat, driving off unreacted nitric acid and oxidizing the remaining organic matter with the perchloric acid. Continue heating, allowing the perchloric acid fumes to condense midway up the neck of the flask, for a half hour after the solution is clear. If a portion of the digest is to be used for total sulfur as well as total phosphorus, add 3 ml of 6 N hydrochloric acid to the cool digest and boil off the water and hydrochloric acid (to fumes of perchloric acid) to remove nitrate as described in the total sulfur method. Small amounts of nitric acid do no harm if only phosphorus is to be determined. Transfer the digest to appropriate size volumetric flask (25 ml to 100 ml), cool, make to volume and mix.

Aliquots containing from 0.0025 to 0.02 mg phosphorus, may be taken for phosphate determination by the stannous chloride method. The aliquots are taken to dryness on the steambath, without need for peroxide treatment, and phosphate determined as described on page 51, starting with the addition of 95 ml of water.

The Amidol Procedure. The amidol method (Allen, 1940) to be described in detail is considerably less sensitive, requiring larger amounts of phosphorus for comparable color intensities, than the stannous chloride method. It, however, possesses the advantage that it is also less sensitive to interferences of various kinds. Timing is not exceedingly critical within the range of 5 to 30 minutes. Reduced sensitivity is usually no problem in total phosphorus determinations, requiring only an appropriate increase in aliquot size from the acid digest.

Transfer an aliquot, not larger than 20 ml and containing not more than 0.4 mg phosphorus, to a 25 ml volumetric flask. On the assumption that 0.2 ml perchloric acid was lost for each 0.5 gram of sample, add sufficient perchloric acid (60 per cent) to provide a total of 2.0 ml perchloric acid. The final amount of perchloric acid may vary as much as 0.2 ml without serious effect on the final color. Add 2.0 ml of the amidol reagent and 1 ml of the ammonium molybdate. Reagents must be added in this order. Make the solution to the mark with water and mix. Within 5 to 30 minutes, transfer the solution to the spectrophotometer or colorimeter cell and read at 660 mμ or with a blue filter transmitting in this region. Prepare appropriate standards in a similar manner.

If the volume of solution required with some colorimeter cells is greater than 25 ml, the volume of the colored solution may be increased taking care to keep the concentrations of reagents the same.

Sulfur (sulfate) by the Methylene Blue Method

Sulfate-sulfur concentrations in dry plant material range from 50 to several thousand parts per million.

Sulfate is determined by reduction to hydrogen sulfide which is swept from the reaction vessel with a stream of nitrogen gas and trapped in zinc acetate solution. The sulfide is reacted with p-amino-dimethylaniline to form reduced methylene blue (colorless). Addition of a solution of ferric iron oxidizes the leuco methylene blue to methylene blue whose intense blue color is measured in a colorimeter. Sometimes the color change goes through a red phase but in 10 minutes only the blue remains. Total time for an analysis for sulfate in a plant sample is about 1½ hours from time of weighing of sample to final measurement of color. With the use of a battery of six distillation units at least thirty determinations per day can be made by an experienced technician. The standard deviation expressed as per cent of the mean is ± 4.9 per cent for sulfate levels of the order of 0.4 per cent in the dry plant materials.

The method distinguishes between inorganic sulfur and organically combined sulfur when the plant sample is treated directly with the reagents (Johnson and Nishita, 1952). Organic sulfur is obtained by difference after determining total sulfur on a wet digest of the material. See page 58 for this digestion method.

Equipment

Cylinder of water-pumped nitrogen, with regulators, etc. Do not use oil-pumped nitrogen.

Micro burners. Alcohol or gas.

Ring stands and clamps.

Digestion-distillation apparatus. This special glassware consists of a boiling flask of 50 ml capacity with a side arm inlet tube for the introduction of nitrogen gas, a water jacketed condenser, an inverted U connecting tube, a gas washing column, and a short piece of detachable connecting tube leading to a volumetric flask. Figure 3.

If many sulfates are to be run, it is convenient to set up a battery of several units. It is also desirable to have extra boiling flasks so that samples may be placed in the flasks while others are being run.

If a battery of units is used a nitrogen gas distributing manifold is necessary. A combination surge bottle and distributing manifold can be made by passing N_2 gas from the cylinder into a large bottle fitted with a rubber stopper containing the required number of outlet tubes. Regulation of rate of flow to individual flasks is accomplished with screw clamps on each connecting tube. *Note:* All rubber stoppers and tubing used must be treated to remove sulfur and zinc contaminants by boiling in two or three changes of 1 N sodium hydroxide followed by thorough washing with sulfur free water.

Frequently rubber tubing has been found to cause erratic results even after this treatment. For this reason, it is now recommended that all tubing used for distribution of nitrogen gas from the cylinder to the manifold and boiling flask be Tygon rather than rubber. Tygon tubing has the disadvantage that it tends to stick to glass. To avoid glass breakage, care must be exercised in removing Tygon tubing from the side tube of the boiling flask.

The delivery tube leading from the gas washing column to the 100 ml volumetric receiving flask is a piece of 4 mm glass tubing 5 inches long. It is connected to the side arm of the gas-washing column with a small piece of rubber tubing (treated to remove sulfur and zinc contaminants). Zinc sulfide adheres to the inside of the glass connecting tube, therefore the tube must be disconnected from the side arm and left in the volumetric flask, until the color has been developed by the addition of the reagents. There

is only a slight error involved if the connecting tube is left in the flask even when the solution is made to final volume.

Reagents

Reducing agent. Mix 300 ml of hydriodic acid (Sp. G. 1.7, methoxyl grade), 75 ml hypophosphorus acid (50 per cent) and 150 ml formic acid (90 per cent). Boil gently with a stream of nitrogen flowing through this solution for about 10 minutes after the temperature has reached 115°C. Do not let the temperature of the solution exceed 117°C. Do not attempt to recover spent reagent by distillation. *Caution:* Extremely poisonous fumes of phosphine (PH_3) may be liberated from the reagent if heated above 120°C or if the reagent is spilled on a hot surface.

Pyrogallol-sodium phosphate wash solution. Dissolve 10 grams of sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$) and 10 grams pyrogallol (pyrogallic acid) in 100 ml of distilled water. Ordinary distilled water should be distilled again from an all-glass still system. Traces of copper in ordinary distilled water will give low results for sulfur. It may also be necessary in some areas to add a small amount of alkaline permanganate to the water at the time of distilling to remove traces of sulfide in the water.

Zinc acetate-sodium acetate. (Sulfide absorbing solution) Dissolve in sulfide-free, copper-free distilled water, 50 grams of zinc acetate dihydrate ($Zn(CH_3COO)_2 2H_2O$) and 12.5 grams of sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$). Make to 1 liter volume and filter if turbid. Solution is facilitated by bubbling a stream of nitrogen gas through the mixture.

Amino dimethylaniline solution. Dissolve 2 grams of p-amino dimethylaniline sulfate in 1500 ml distilled water. Add to this solution 400 ml of concentrated reagent grade sulfuric acid. Make the cooled solution to 2 liters.

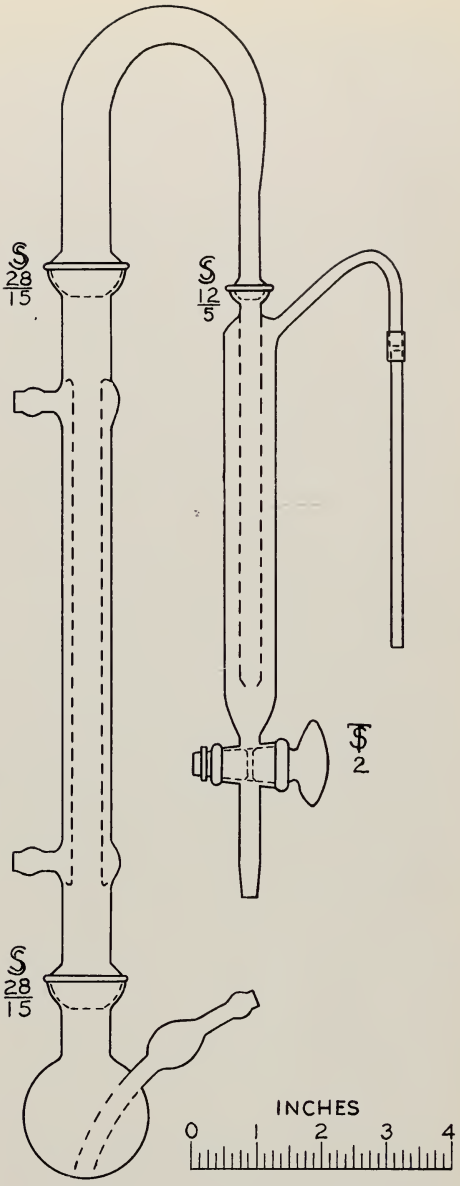


Fig. 3. Detail of construction of glassware for sulfate method.

Ferric amomnium sulfate solution. To 25 grams of ferric ammonium sulfate [$Fe_2(SO_4)_3 \cdot (NH_4)_2SO_4 \cdot 24H_2O$] add 5 ml of concentrated sulfuric acid and 195 ml distilled water (in this order). The salt dissolves slowly often requiring two or three days with frequent shaking.

Standard sulfate solutions: *Stock solution*, containing 1 mg sulfate *sulfur* per milliliter. Dissolve 5.434 grams of reagent grade potassium sulfate (K_2SO_4) in sulfur-free distilled water and make to 1 liter.

Working solutions. Suitable working solutions are prepared by dilution of aliquots of the stock solution with sulfur-free distilled water. For example, one milliliter of the stock solution diluted to one hundred milliliters with sulfur-free distilled water will provide a working solution such that 1 ml contains 10 micrograms of sulfate *sulfur*. Usually a series of working solutions are prepared so that 1 ml contains 2, 10, 20, 50 and 100 micrograms sulfate *sulfur*. Choice of amount of sulfate *sulfur* depends on the kind of colorimeter used and the expected amounts of sulfate *sulfur* in the sample. It is desirable to use standards containing approximately the same amount of sulfate as in the sample. After some experience with the method, especially after noting the amounts of sulfur for which a linear relationship between color and amount of sulfur is obtained, it is necessary to run only one or two standard amounts with each lot of samples.

Do not run standards or samples containing more than 200 micrograms of sulfur. Large amounts of sulfur, aside from giving too highly colored solutions to read accurately, will sometimes cause deposition of elemental sulfur in the apparatus.

Sulfur-free ground joint lubricant. Most ground joint lubricants contain appreciable sulfur which must be removed before use. Many lubricants deteriorate quickly when exposed to the hot reducing agent. Dow-Corning Silicone stopcock lubricant has been found suitable if freed of sulfur contaminant. Place about 5 grams of the silicone lubricant in a 100 ml beaker, add 10 ml of an equal volume mixture of the hydriodic acid and hypophosphorus acids (the formic acid is unnecessary in this procedure and produces obnoxious fumes, so is omitted). Fill one of the 50 ml boiling flasks with cold water and set on top of the beaker to act as a condenser. Boil the mixture gently with frequent stirring for about 45 minutes. Pour off the acid mixture and wash the lubricant thoroughly with sulfur-free distilled water.

Procedure

Lubricate all spherical joints with a minimal amount of the treated lubricant. Place 10 ml of the pyrogallol-sodium phosphate solution in the gas washing column of the digestion-distillation apparatus.

Place 10 ml of the zinc acetate-sodium acetate solution in a 100 ml glass stoppered volumetric flask, and add approximately 70 ml sulfur-free distilled water. Connect the delivery tube to the side arm of the gas washing column and clamp the receiving flask in place. The delivery tube should be long enough to reach nearly to the bottom of the receiving flask.

Transfer an aliquot of the standard or liquid sample to the boiling flask. The volume of liquid sample used should not exceed 2 ml. If it is necessary to use larger aliquots, reduce the volume by evaporation.

Sulfate-sulfur in dried plant material is determined by transferring, directly, 25 mg of the ground sample and adding 2 ml of sulfur-free water and proceeding as described for standards and liquid samples.

Add 4 ml of the reducing agent (hydriodic, hypophosphorus, formic acid mixture). Attach the distillation flask to the condenser and connect the tube from the nitrogen supply and adjust the nitrogen flow rate so that about 2 bubbles per second issue from the receiving flask. This rate is not extremely critical. Make certain that cool water is flowing through the condenser. After about 5 minutes of nitrogen flow, light the microburner, and with nitrogen continuing to flow main-

tain the contents of the distillation flask at a low boil for 1 hour.

Remove the receiving flask, leaving the connecting tube in the zinc acetate solution. Using a rapid delivery pipette, add 10 ml of the p-aminodimethyl-aniline solution. Quickly stopper the volumetric flask and mix thoroughly, add 2 ml of the ferric ammonium sulfate solution, mix again, then make to volume and mix thoroughly. No appreciable error is introduced by leaving the connecting tube in the flask at this point.

The methylene blue color is stable and may be read after 10 minutes but within 24 hours after development.

If a prism or grating spectrophotometer (Beckman or Coleman) is available make the readings at a wavelength of 670 mμ.

If a filter type colorimeter is used, select a filter which transmits as closely as possible to this wavelength. The red filter used for reading the molybdenum blue color in phosphorus determinations is usually adequate. It should be noted that with a filter colorimeter the linear relationship between reading and amount of sulfur holds over a shorter range than is the case with a prism or grating spectrophotometer.

Calculation

$$\frac{\text{R sample}}{\text{R standard}} \times \text{micrograms } S \text{ in standard} \times \frac{1000}{\text{mg of sample}} = \text{ppm } SO_4^{=} - S$$

R sample = colorimeter reading for sample corrected for blank reading.

R standard = colorimeter reading for standard corrected for blank reading.

Blank readings are obtained by using 2 ml of sulfur-free distilled water in place of sample or standard. The blanks are usually very small but should be run frequently to detect inadvertent sulfur contamination of reagents and glassware.

If the methylene blue color is too intense to read, make appropriate dilution with a solution containing 10 ml of the p-aminodimethylaniline solution and 2 ml of the ferric ammonium sulfate solution in 100 ml sulfur-free distilled water. Dilution with distilled water alone causes changes in color intensity which render the readings useless.

Sulfur (total) by the Methylene Blue Method

Amounts of total sulfur in plant material range from 1,000 to 15,000 ppm.

Total sulfur in plant materials is determined by the methylene blue method after oxidation of the sample by any one of several oxidation methods, including wet digestion with nitric and perchloric acids, the A.O.A.C. magnesium nitrate method, and the Parr peroxide bomb method.

Wet digestion with nitric and perchloric acids is the simplest of the methods and will be described in detail. The sample is predigested with nitric acid for 30 minutes to destroy easily oxidizable matter and to oxidize any sulfide-sulfur arising from acid hydrolysis of organic sulfur compounds. After the predigestion the remainder of the organic matter is oxidized with perchloric acid. Excess nitric acid must be removed because of interference of nitrate in the final step of the determination. Nitric acid is removed from the digest by adding an excess of hydrochloric acid and boiling.

Procedure

Depending on the amount of total sulfur expected in the plant material, transfer 100 mg or less to a 30 ml micro-Kjeldahl flask, add 2 ml of concentrated

nitric acid, and digest on the steam bath for 30 minutes. Add 1 ml of 60 per cent perchloric acid and heat slowly over a microburner. As the digest clears up increase the heat and continue the digestion for an hour after fumes of perchloric acid begin to condense in the neck of the flask. The micro-Kjeldahl flasks should be shielded so that free flame from the burner does not play directly on the surface above the liquid level, otherwise a loss of sulfuric acid may result. Cool the flask, add 3 ml of 6 N hydrochloric acid. Boil off the water and hydrochloric acid. The perchloric acid is not boiled off; sufficient heat is applied only to cause the perchloric acid fumes to condense about midway in the neck of the flask. Cool, transfer to a 50 ml volumetric flask, make to volume and mix. Take aliquot, usually 2 ml for determination of sulfur by the methylene blue method.

Aliquots larger than 5 ml should not be used because of possible interference from the amount of perchloric acid which is associated with this size aliquot.

DETERMINATION OF THE MICRONUTRIENT ELEMENTS

The term micronutrient elements refers to seven elements, which are required by plants in relatively small amounts. These, in the approximate order of concentrations required by healthy plant tissues, are chlorine (Cl), iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu) and molybdenum (Mo). The amounts in healthy tissues, although sometimes variable for different species, range from 100 ppm for chlorine to 0.1 ppm for molybdenum, although several times these amounts often are found in tissues from field-grown plants.

Collection and Preparation of Samples

Extraordinary care must be taken to avoid spurious analytical results for many of the micronutrient elements arising from dust, soil, fertilizer and spray residue contamination. (Generally the error from these sources is not serious for macronutrients, with the possible exception of calcium contamination from very calcareous or gypsiferous soils.) The concentrations of the micronutrient elements in soils are usually in excess of those found in normal plant materials. Soil contamination therefore is certain to result in high and extremely variable results for most micronutrients. Spray residues (Bordeaux, etc.) will also invalidate analytical results for copper, zinc and other of the metal micronutrients. Many workers (Hammer, 1956; Jacobson and Oertli, 1956; Smith, Reuther, and Specht, 1950) have presented data illustrating effects of dust and spray residues on leaf analysis and all agree that analyses of unwashed plant samples for the micronutrients are highly questionable. General recommendations include washing of individual samples by washing with a detergent or detergent acidified with 0.1 N hydrochloric acid followed by thorough rinsing of the fresh material with distilled water. The samples are then dried in a forced draft oven at 70–80°C and ground as described below. At all times the samples should be protected from dust contamination by sealing in dust-proof containers. Samples should be dried in the original bags or clean aluminum pans to prevent contamination from brass or iron shelves in the drying ovens. A recent report indicates that paper bags may contain considerable boron which may be transferred to moist samples (Winsor, 1957).

Most types of grinders contribute some contamination of the micronutrient elements. Special care must be taken to avoid the use of grinding mills with brass parts (zinc and copper contamination) or with soft iron parts in the case of iron

determinations. Grinding of material for zinc and copper determinations should be done only in Wiley mills with stainless steel screens and steel cutting blades with no trace of brass coming in contact with the samples. Manganese and cobalt determinations may also be done on these samples. Iron determinations may be done successfully only on samples ground in a mortar or hand crushed. Often the best method of preparing leafy material is to hand crush the thoroughly dry sample in the original bag. Obviously, stemmy material cannot be so successfully crushed in this manner, but if enough material is available so that a large sample—say 5 to 10 grams—may be taken for the ashing, the degree of fineness is not so important. In this case even stemmy material may be broken up sufficiently fine to permit adequate sampling.

TERMINOLOGY

Microgram (Symbol γ) = millionth of a gram; thus one microgram of a substance per gram of sample is one part per million. For instance, 100 micrograms of iron per gram of dry plant material is equivalent to 100 ppm of iron

1 per cent of a substance equals 10,000 ppm.

"Trace elements" and "minor elements" are terms sometimes used in the literature and in conversation for micronutrient elements. Both these terms have the false implication that micronutrients are less important to plants than macronutrients. The term "trace element" is also inappropriate as chemists can now exactly measure the concentrations of the micronutrient elements and no longer need, as they did several years ago, report the presence of "traces" of elements.

Glassware and Reagents for Micronutrient Analysis

All flasks, beakers, reagent bottles and other vessels used for micronutrient analysis should be reserved for this use only and never used in macronutrient analysis procedures where large amounts of some of the micronutrients may be used as reagents, as for example, copper in macro-Kjeldahls, manganese in calcium determinations, lead in sugar determinations, etc.

All glassware used in micronutrient analyses must be of borosilicate glass, usually obtainable as Pyrex or Kimax brand. Special glassware requirements for boron analyses will be discussed in the section on boron determination. Prior to each use, the glassware should be washed with warm 3 N hydrochloric acid, rinsed with several small portions of redistilled water and then rinsed with a 10 per cent solution of ammonium ethylenediaminetetraacetate [$(NH_4)_3EDTA$] adjusted to pH 8 and finally washed with redistilled water.

Distilled water as ordinarily produced in the laboratory may contain appreciable amounts of heavy metals, copper, zinc, and often molybdenum. This water should be redistilled through an all-glass still and condenser system into borosilicate bottles. Frequent blank determinations of glassware, reagents and water should be made.

Storage of distilled water and reagents for use in spectrophotometric determinations in polyethylene containers is not recommended. Thin films, possibly incompletely polymerized polyethylene, deposit on separatory funnels and spectrophotometer cells. These films reduce the accuracy of the determinations and are difficult to remove.

While analytical chemists in the past have recommended that acids and some solvents be especially distilled for use in micronutrient analyses, this precaution is now generally unnecessary provided reagents labelled "A.C.S. grade—suitable for microanalysis" are used and blank determinations are run on each new lot

of reagents. Distillation of sulfuric, nitric and hydrochloric acids is tedious and attended by considerable danger. Distillation of perchloric acid may be exceedingly dangerous and should not be attempted except in laboratories where special equipment and personnel are available. Likewise redistillation of organic solvents is generally unnecessary and may be hazardous unless done with special regard for fire hazards or for disposal of toxic fumes. Use of high quality acids and solvents, obtainable at only negligible additional cost, eliminates any need for time consuming and hazardous distillations in laboratories.

Dithizone as a Reagent for Micronutrients

Dithizone is one of the most versatile organic reagents for micro amounts of many heavy metals and its importance in separations and estimations of elements of interest in soil and plant analysis warrants a fairly detailed discussion. The reader is referred to Sandell (1950) for a comprehensive account of dithizone chemistry.

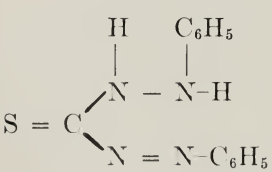
Dithizone, more properly called diphenylthiocarbazone, is a violet-black solid, insoluble in water at acid and neutral pH values, but soluble in many organic solvents, most notably carbon tetrachloride and chloroform.

The formulae of the acid and of the zinc salt (see below) are usually abbreviated to Dz and $Zn(Dz)_2$ and it is implied that dithizone is a weak acid and dissociates according to the equation $Dz \rightleftharpoons Dz^- + H^+$. Dithizone (acid form) is virtually insoluble in water but addition of a base, for example, ammonium hydroxide, results in the formation of ammonium dithizonate which is very soluble in water giving an intense yellow to orange color, $Dz + NH_4OH \rightarrow Dz^- + NH_4^+ + H_2O$. The acid dithizone dissolves in carbon tetrachloride to the extent of 0.05 gram per 100 ml and in chloroform the solubility is 2 grams per 100 ml, in both cases giving a green solution. Normally, in spite of the lower solubility of dithizone, carbon tetrachloride solutions of dithizone are used. The solubility of dithizone in carbon tetrachloride is ample for most purposes and in fact confers some advantages. Chloroform as a solvent and extractant has the disadvantage that the partition of dissociated and undissociated dithizone between the alkaline aqueous phase and the chloroform is much more in the direction of the organic phase than is the case with carbon tetrachloride. For example, the distribution ratios between 1:200 ammonium hydroxide and the organic solvents have been given as:

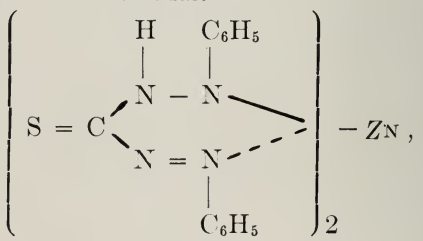
$$\frac{(Dz^-)NH_4OH}{(Dz)CCl_4} = 41 \quad \text{and} \quad \frac{(Dz^-)NH_4OH}{(Dz)CHCl_3} = 1.6$$

This means that most of the dithizone is in the alkaline aqueous phase with carbon tetrachloride as extractant and there is very little of the green color of unreacted dithizone (Dz) in the carbon tetrachloride to mask the pink color of the metal dithizonate, thus making it relatively easy to be certain that all metals have been extracted into the car-

acid form



zinc salt



bon tetrachloride phase. On the other hand if chloroform is the extractant, there is so much unreacted dithizone in the chloroform layer that it is difficult to detect the color of metal dithizonates.

Dithizone reacts with a number of heavy metals including manganese, iron, cobalt, nickel, copper, zinc, palladium, silver, cadmium, indium, tin, platinum, gold, mercury, thallium, lead and bismuth. Some of the metals react with dithizone in only one valence state. For example, ferrous but not ferric, stannous but not stannic, platinous but not platinic ions, give colors with dithizone. Some of the metals, for example, manganese, react only under special conditions and form unstable compounds of no value in analytical chemistry. Dithizone reactions can be made quite specific for some elements, by proper adjustment of pH, by adding complexing agents which will tie up other reacting metal ions, or sometimes by changing the valence state of interfering ions. For example, the two dithizone reacting metals found in largest amounts in plant materials, zinc and copper, can be separated by adjustment of pH. Both copper and zinc react with dithizone at pH 8.5–9.0 and are extracted into the carbon tetrachloride phase. If the mixed dithizonates in the carbon tetrachloride are shaken with dilute hydrochloric acid (pH about 2) the zinc dithizonate is decomposed and zinc ion is quantitatively transferred to the acid solution but the copper dithizonate is stable at this pH and remains in the organic phase. Complexing agents, especially citrates, are often added not only to render the dithizone reaction more specific but also to prevent precipitation of heavy metal phosphates, carbonates, and hydroxides in the alkaline pH range. Citrate also prevents precipitation of calcium and magnesium salts. Precipitates are objectionable because they are certain to adsorb heavy ions and prevent quantitative extraction of the dithizonates into the carbon tetrachloride. Ferric ion unless complexed in some way, interferes by oxidizing the dithizone as well as by forming undesirable precipitates.

Under prescribed conditions, the heavy metals may be estimated by measurement of the absorbance of the carbon tetrachloride solutions of the dithizonates. The only heavy metal of interest in plant analysis that is determined directly in this way is zinc. The other metals are determined by more specific and accurate methods, often after a preliminary separation from the bulk of the ash solution by extraction of the dithizone reacting metals, as for example, in the determination of cobalt. The preliminary separation with dithizone serves two purposes, first and most importantly, separation from interfering elements and secondly, concentration of the element in a small volume.

Dithizone is easily oxidized to the diazone which is insoluble in aqueous solutions but dissolves in carbon tetrachloride and chloroform to give a yellow solution which leads to errors in quantitative work. Some dithizone available commercially may contain the oxidation product which does no harm if dithizone is used solely for preliminary separation of heavy metals from the ash solution. However, when zinc is determined quantitatively by the dithizone procedure, it is necessary to purify the material. Purification is based on the insolubility of the diazone in ammoniacal solutions. The carbon tetrachloride solution of dithizone is shaken with several successive portions of 1:100 ammonium hydroxide, the oxidized dithizone remaining in the carbon tetrachloride phase which is discarded. The aqueous phase is acidified with hydrochloric acid (color changes from orange-yellow to blue or purple) and extracted with carbon tetrachloride. The purified solution may be kept in a dark bottle and in a cool place (preferably in a refrigerator) for some time without deterioration.

The separatory funnel shaker shown

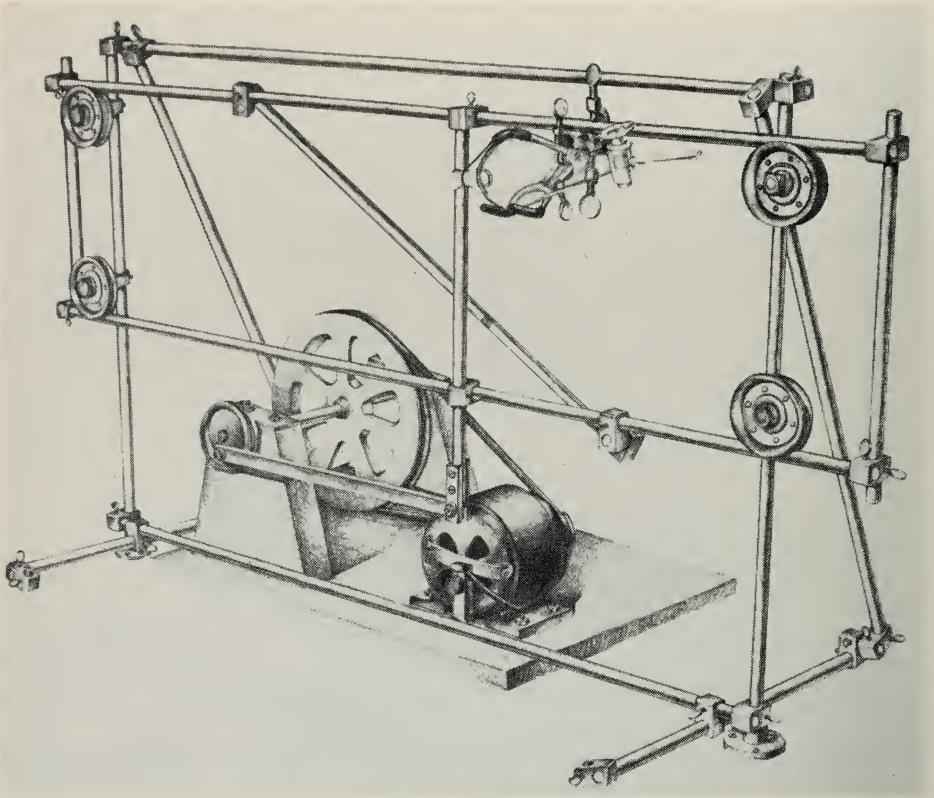


Fig. 4. Separatory Funnel Shaker. The shaker is constructed from standard ½" diameter aluminum alloy rods and connectors. The oscillating frame rolls on two pairs of 2⅞" pressed steel ball bearing sheaves fastened on the stationary frame work.

The shaker is powered by a 1/12 horsepower motor. Pulley sizes are selected to give about 250 oscillations of the frame per minute with a 1½" stroke.

Large size 3-prong laboratory clamps are used to hold separatory funnels firmly. Stoppers and stopcocks should be seated firmly with a slight twisting motion. No clamps are needed to hold stoppers in place.

The shaker may be used to shake a variety of vessels, such as soil extraction bottles, centrifuge tubes, etc.

Design and construction of shaker by T. H. Arkley and A. B. Carlton.

in Fig. 4 greatly facilitates analytical work involving extractions with immiscible solvents, as for example in the determination of copper (page 65), molybdenum (page 69), and zinc (page 75).

Determination of Boron

Boron concentrations in plants may vary over wide ranges; values as low as 5 ppm for deficient plants have been reported. Normal ranges appear to be from 10 to 100 ppm depending on species and supply. Values as high as 1,000 ppm have been reported for plants suffering under boron toxicity.

Probably the most satisfactory method for small amounts of boron is that of Dible, Truog and Berger (1954) which will be described with only slight modification.

Because of the volatility of boric acid,

wet ashing methods for boron cannot be used. Some workers recommend the addition of a base like calcium hydroxide prior to dry ashing to prevent the loss of boron. However, a number of workers have shown conclusively that most vegetative tissues contain sufficient bases to prevent the loss of boron when the sample is ashed at 550°C. Only in the case of seeds where there is a possibility of excess anions over cations is it necessary to use additional base when ashing. In no event should acid such as alcoholic sulfuric acid be added before ashing.

Equipment

Boro-silicate glass formulations as used in heat resistant glassware (Pyrex or Kimax brands) are not satisfactory because they release appreciable amounts of boron to solutions which are stored or heated in them. Boron determinations in the micronutrient range therefore must be made in other types of laboratory ware such as porcelain, platinum or preferably in special low boron glassware available for this purpose. Reagents usually may be safely stored in soft glass bottles although blank determinations should be used to check boron contamination whenever new lots of reagents are used.

Reagent bottles, volumetric flasks and pipettes of soft glass or other non-borosilicate glass. (Corning Brand No. 7280 glass is suitable.)

Small (approx. 2" diameter) crucibles or evaporating dishes. Porcelain, quartz or platinum are satisfactory.

Water bath with controls to provide a temperature of 55± 3°C. This temperature control is critical.

Spectrophotometer or colorimeter with filter transmitting at 540 mμ.

Reagents

Curcumin-oxalic acid solution. Dissolve 0.04 grams of finely ground curcumin and 5 grams of oxalic acid $(COOH)_2 2H_2O$ in 100 ml of 95 per cent ethyl alcohol. If stored in a dark bottle in the refrigerator, this reagent will keep satisfactorily for a week.

Ethyl alcohol. Good quality. 95 per cent.

Hydrochloric acid, 1.0 N. Dilute 83 ml of concentrated hydrochloric acid to 1 liter with distilled water.

Standard boron solutions. Dissolve 0.286 grams of boric acid (H_3BO_3) in distilled water and transfer to a 1,000 ml volumetric flask, make to the mark and mix. One ml of this solution contains 50 micrograms of boron. Prepare a series of solutions containing 0.1, 0.25, 0.5, 1.0, and 2.0 micrograms boron per milliliter for use in setting up a standard curve. It is best to have the dilute stock solutions of such strength that 1 ml aliquots of each solution may be taken directly for color development. Boric acid solutions cannot be evaporated on the steam bath without loss unless the solution is made alkaline with calcium hydroxide. Sodium salts depress color formation, therefore do not use sodium hydroxide to make solutions alkaline prior to evaporation.

Procedure

Preparation of standard curve. Place 1 ml aliquots of each of the standard solutions, including a blank using distilled water, in 250 ml boron-free glass beakers. One milliliter aliquots are critical and must be strictly adhered to. Porcelain evaporating dishes may be used instead of the beakers provided the dishes are treated with a material such as Dessicote to prevent creeping of the treated sample. Add 4 ml of the curcumin-oxalic acid solution and mix thoroughly. Place the vessels in a water bath maintained at 55± 3°C and evaporate to dryness. Keep the dried residues at this temperature for at least 15 minutes. Temperature control within the limits noted and thorough drying of the residue are critical features of the method. Cool the vessels and residue to

room temperature and add exactly 25 ml (volumetric pipette) of 95 per cent ethyl alcohol. Rub the residues with a rubber policeman to insure complete extraction of the colored compound by the alcohol. Clarify the extract by centrifuging, or filtration through close textured filter paper (Whatman No. 2) or by allowing to stand until the solution is clear. Avoid evaporation losses. Transfer the alcoholic solution to appropriate spectrophotometer cells and read the absorbance at 540 mμ. Prepare a plot of absorbance versus micrograms of boron taken through the procedure.

Boron determination in plant tissue. Transfer 0.25 to 0.50 grams of the ground sample to a porcelain, quartz or platinum dish and ash in the muffle furnace at 550°C to a graywhite ash. Usually 2 to 4 hours are sufficient. Cool the dishes, moisten the ash carefully with a bit of distilled water and add 5 ml of 1.0 N hydrochloric acid. Transfer the ash solution, without filtering, to a suitable size soft glass volumetric flask, make to the mark with washings of distilled water and mix thoroughly.

The dilution of the ash solution depends on the expected content of boron in the sample. Usually 25 ml is satisfactory but the final volume should ideally be such that a 1 ml aliquot will contain an amount of boron that falls in the useful range of the standard calibration curve. The 1 ml aliquot of aqueous sample is critical and must not be exceeded. Develop and measure the color as described under the preparation of standard curves. The color fades slowly, therefore, color reading should be made within one or two hours after development.

Calculate and report the results in terms of micrograms of boron per gram (ppm) dry weight of the sample.

Determination of Copper

Plant samples may contain from 1 to 20 ppm of copper.

Of the two methods, dithizone and carbamate methods, commonly used for the colorimetric determination of copper in soils and plant materials, the carbamate method finds the most favor. The dithizone methods, while the most sensitive, require more attention to fine detail and are subject to considerable error from interfering ions. The carbamate methods, especially as recently modified (Cheng and Bray, 1953; Forster, 1953) are rapid, free from interferences and are sufficiently sensitive for use in plant analysis.

Both methods depend on the use of ethylenediaminetetraacetate [EDTA] to chelate interfering cations and thus prevent the development of spurious colors with carbamate. The EDTA chelate of copper is less stable than is the copper carbamate and thus the full color of the copper carbamate is extractable with chloroform or carbon tetrachloride.

Equipment

Micro digestion unit, with borosilicate glass fume pipe.

Separatory funnels, 120 ml Squibb, pear shaped, with tip cut off to 1″ length. A separatory funnel shaker (Fig. 4) is useful but not required.

Colorimeter or spectrophotometer. For highest sensitivity and accuracy a spectrophotometer with a grating or prism is required. The use of filter photometers results in lower sensitivity, shorter range over which linear relationship between concentration of the determined element and instrument reading is obtained and also a greater possibility of interferences from elements which might not be of consequence when a spectrophotometer is used.

Reagents

Copper standard, stock solution —100 ppm copper. Dissolve 0.393 grams of copper sulfate pentahydrate [$CuSO_4 \cdot 5H_2O$] in redistilled water in a liter volumetric flask, add 5 ml of concen-

trated sulfuric acid. Make to volume when cool and mix.

Copper standard, working solution. 5 ppm copper. Prepare fresh daily. Dilute 5.00 ml of the stock solution with redistilled water in a 100 ml volumetric flask and mix. One milliliter of this solution contains 5 micrograms of copper. Standard curves are established by taking 0, (blank), 1, 2, 5 and 10 ml of this working solution through the procedure and plotting spectrophotometer readings against micrograms of copper taken.

Ammonium ethylenediaminetetraacetate. Suspend 250 grams of ethylenediaminetetraacetic acid [EDTA] in 500 ml redistilled water, add 200 ml of concentrated ammonium hydroxide, stir until dissolved, filter and make to 1 liter volume.

Ammonium hydroxide 1:1. Dilute concentrated ammonium hydroxide with an equal volume of redistilled water.

Sodium diethyldithiocarbamate solution. Prepare fresh at least weekly and store in a cool, dark place. Dissolve 1 gram of the salt in 100 ml of redistilled water.

Carbon tetrachloride. A.C.S. grade.

Procedure

Ashing of plant material. Wet ashing of plant material for copper determination is preferred. Dry ashing is bound to result in low analytical values for copper because of retention of copper by the insoluble silica. If dry ashing must be done, it is necessary that the silica be removed by treatment of the ash (in a platinum dish) with hydrofluoric acid. This procedure is time consuming and requires the use of platinum rather than the more easily available borosilicate glassware (Pyrex or Kimax brands). In addition, there is added risk of introducing variable amounts of copper contaminants from hydrofluoric acid. In either ashing procedure, samples that are high in calcium may cause trouble when sulfuric acid is present by precipitating calcium sulfate which adsorbs copper strongly. For this reason, as well as others, the wet ashing procedure described here avoids the use of sulfuric acid in the wet ashing. Provided the sample size does not exceed 1.00 gram and at least 10 ml of concentrated nitric acid and 2 ml of perchloric acid are present at the start, it is possible to carry out the wet ashing with nitric and perchloric acid without the usual sulfuric acid being present.

Transfer 1.00 gram of dry sample to a 30 ml micro-Kjeldahl flask and wet ash with nitric and perchloric acids as described on page 32. Cool the contents of the digestion flask and transfer directly to a 120 ml separatory funnel, previously marked at the 60 ml level. Rinse the digestion flask with 5 ml of the ammonium EDTA solution and transfer to the separatory funnel. Do not exceed the 60 ml volume when adding the rinsings from the digestion flask. Add three drops of phenolphthalein and add sufficient of the 1:1 ammonium hydroxide to give a faint pink color.

Note: a precipitate of EDTA may form when the NH_4 EDTA is added to the acid solution. This precipitate will dissolve when the ammonium hydroxide is added. Add exactly 2.5 ml of the carbamate solution and exactly (with a volumetric pipette) 10.00 ml of carbon tetrachloride. Shake the separatory funnel vigorously for at least two minutes, venting once or twice by inverting the funnel and opening the stopcock. Allow the phases to separate as sharply as possible. Dry the tip of the funnel, using absorbent cotton or lens tissue or by applying vacuum through a fine tipped tube. Insert a small piece of absorbent cotton in a small clean funnel and filter a portion of the carbon tetrachloride solution directly into the spectrophotometer vessel. The spectrophotometer vessel should have been previously rinsed with carbon tetrachloride and allowed to drain dry. The filtration through absorbent cotton is for the pur-

pose of removing traces of water which would lead to erroneous readings in the spectrophotometer.

Read in the spectrophotometer at once, using a wavelength setting of 440 mμ and carbon tetrachloride as the reference solution. If a filter photometer is used, a filter transmitting in the blue near 440 mμ should be selected. The carbon tetrachloride solution of copper carbamate tends to fade, especially in sunlight, therefore the solution should be protected as much as possible from light and read promptly after extraction.

Calculations

From the plot of standard copper taken against spectrophotometer reading obtain the amount of copper in the unknown and report as micrograms of copper per gram of sample (ppm) taken through the procedure.

Determination of Iron

Iron contents of plant materials are extremely variable, ranging from 10 to several hundred parts per million. Exceptionally high results should be viewed with suspicion of soil contamination, or contamination from grinding mills. Samples for iron analysis should be hand crushed rather than risk contamination from grinding mills.

There are many methods for the determination of iron with advantages claimed for each (Sandell, 1950). However, the most satisfactory seems to be the o-phenanthroline method because the color forming reaction can be carried out under slightly acid conditions so that hydroxides, phosphates and carbonates of many metals occurring in plant samples are not precipitated.

Ferrous iron reacts with o-phenanthroline in solutions of pH 2–6 to form a stable orange-red complex. It is necessary to reduce all of the iron from the plant ash to the ferrous state. This is best done with hydroquinone in the presence of citrate.

Equipment

Wet digestion unit. See page 30.

Micro-Kjeldahl flasks. 30 ml or 100 ml.

Spectrophotometer or colorimeter.

Reagents

Hydroquinone, 1 per cent solution in water. Freshly prepared daily.

Sodium citrate, 250 grams per liter of aqueous solution.

o-Phenanthroline, 0.5 per cent solution of the monohydrate salt in water. It may be necessary to warm slightly to aid in solution. The solution should be kept in the dark and discarded if any color develops.

Ammonium hydroxide, 1:1. Dilute concentrated ammonium hydroxide with an equal volume of water.

Standard iron stock solution. Dissolve 0.702 grams ferrous ammonium sulfate [$FeSO_4(NH_4)_2SO_46H_2O$] in 50 ml redistilled water containing 10 ml concentrated sulfuric acid in a liter volumetric flask. When cool make to mark and mix thoroughly. 1 ml of this solution contains 100 micrograms iron.

Dilute iron standard. Dilute 10 ml of the stock standard to 100 ml. One ml of this standard contains 10 micrograms iron. (Pure iron wire may also be used as a standard.)

Preparation of standard iron calibration curves

Transfer to 25 ml volumetric flasks, aliquots of the standard iron solutions to provide 0, 5, 10, 20, 40, 80 and 100 micrograms of iron. It will be necessary to use the dilute standard for the 5 and 10 micrograms and the stock standard for the higher concentrations. Use redistilled water for the 0 (blank) level. Add 1.0 ml of the o-phenanthroline solution, 1.0 ml of the hydroquinone, 5 ml of the sodium citrate solution and sufficient of the 1:1 ammonium hydroxide to give a

pH of 3.5–4.0. Use a pH meter to indicate proper amount of ammonia to add. The reagents must be added in this order and care should be taken to avoid excessively high local concentrations of ammonium hydroxide because the hydroquinone rapidly oxidizes above pH 6, and gives an off-brown color to the solution.

The sodium citrate provides considerable buffering in this range and aids in attaining proper pH control.

Dilute the colored solution to the mark, mix and allow to stand 1 hour. Measure the absorbance of the iron-phenanthroline complex at 508 mμ or, if using a filter photometer, with a filter showing maximum transmission in the range 480–520 mμ. Plots of absorbance versus amount of iron should be linear.

Iron determination in plant sample

Wet digestion of the material (as described on page 32) is preferred. Dry ignition of the material is permissible only if precautions are taken to remove silica, a process requiring use of platinum ware and hydrofluoric acid. Transfer the cooled digest to a 50 ml beaker, add 2 drops of concentrated sulfuric acid and take to dryness on steambath. Fume off the perchloric acid by heating on a hot plate or over a Meeker burner. Perchloric acid must be removed because it inhibits o-phenanthroline color development. To the cool beaker, add 10 ml of N hydrochloric acid and warm on the steambath to hydrolyze pyrophosphate which formed during the fuming step. Proceed with the determination as described in this bulletin for the standard curves.

The amount of iron in the aliquot used is obtained by reference to the standard curve. The iron concentration in the sample then is calculated taking into consideration the sample weight, the volume of the ash solution and aliquot taken to obtain a reading in the useful range of the standard curve.

Determination of Manganese

Manganese concentrations in plant material may vary from approximately 10 ppm to several hundred ppm. In some instances there may be sufficient manganese in a plant sample to give a transitory pink solution when the ash is acidified or a pink color may develop in wet digestions of such material.

The most sensitive and accurate chemical method for manganese is based on oxidation to permanganate whose pink color is very stable if protected from dust and reducing fumes. (Sandell, 1950).

Dry ashing of plant material for manganese determination is not regarded as suitable because of the tendency of manganese to be held in insoluble form by the siliceous residue of the ash. In order to obtain accurate results from dry ashing it is necessary to remove the siliceous material by treatment with hydrofluoric acid, a tedious procedure which requires the use of platinum dishes as well as the hazardous hydrofluoric acid. There is no retention of manganese on the silica residue obtained from wet ashing and the wet digestion method is the only one recomended for manganese determinations.

Equipment

Wet digestion apparatus—see page 30.

Spectrophotometer or colorimeter.

Reagents

Nitric acid, concentrated.

Sulfuric acid, concentrated.

Perchloric acid—60 or 72 per cent.

Phosphoric acid 85 per cent.

Potassium meta-periodate, KIO_4.

Sodium sulfite, Na_2SO_3.

Manganese Standard Solution. Dissolve 0.288 grams pure dry potassium permanganate in 300 ml of water in 1,000 ml beaker and add 20 ml concentrated sulfuric acid. Add solid sodium sulfite until the pink color of the permanganate is just discharged. Boil gently to remove the excess sulfur dioxide. Cool and transfer to a liter volumetric flask with wash-

ing, make to the mark and mix. This solution contains 100 micrograms of manganese per milliliter. Prepare a more dilute working standard by diluting 100 ml of the stock standard to 1 liter. One milliliter of this solution then contains 10 micrograms of manganese. If the purity of the original solid potassium permanganate is in doubt the concentration of the permanganate solution before reduction with sulfite should be checked by titration against standard sodium oxalate.

Procedure

Preparation of standard curve. Transfer to 100 ml beakers appropriate known amounts of manganese from the working standard solution (i.e. 0, 10, 20, 40, 80, 150, 500 micrograms Mn) and add to each beaker 2 ml of 85 per cent H_3PO_4, 2 ml concentrated sulfuric acid, and sufficient water to make a total volume of about 25 ml. Add 0.3 grams of potassium periodate and two boiling beads. Cover the beakers with a watchglass. Bring to a gentle boil and maintain the gentle boil for about 5 minutes after development of color. When cool, transfer quantitatively to a 50 ml volumetric flask, make to volume and mix. Read the absorbance at 525 mμ in a spectrophotometer or colorimeter. Prepare a plot of absorbance reading, corrected for the blank which is usually very small, versus micrograms of manganese taken.

Determination of manganese in plant sample. Transfer an amount of plant material likely to contain from 50 to 500 micrograms of manganese—usually from 1 to 5 grams of dry sample—to a 300 ml Kjeldahl flask. Ash the sample with nitric and perchloric acid as described on page 32. Continue digestion until the flask is filled with dense white fumes of perchloric acid, and until most of the perchloric acid is fumed off, leaving only a moist residue of salts.[4]

When the digest has cooled add 2 ml of 85 per cent phosphoric acid and 25 ml distilled water. Filter through a 9 cm Whatman No. 44 filter and wash flask and paper thoroughly with hot water. Reduce the volume of the filtrate to about 25 ml, add 1 or 2 glass beads, or a Hengar chip, and about 0.3 grams of sodium or potassium periodate. Bring to boil and keep at a gentle boil for two or three minutes for maximum color development. If much chloride is present, color development may be delayed because of reduction of permanganate by chloride. It may be necessary to add more periodate and prolong the heating period. Chloride interference may be detected by noting the odor of chlorine gas above the boiling solution. Cool, transfer to 50 ml volumetric flask and make to volume with distilled water that is free of reducing substances. If the permanganate color appears to fade on dilution with distilled water, place the flasks in a boiling water bath for about one half hour to restore the full permanganate color. Distilled water that is free of reducing substances may be prepared by distilling from a glass still water containing a trace of permanganate and potassium hydroxide.

After cooling, make to volume, mix and measure the absorbance of the solution at 525 mμ. The amount of manganese in the sample then can be obtained from the standard curve.

Determination of Molybdenum

Molybdenum concentrations in plants may be as low as 0.02 ppm in some deficient plants and as high as 20–50 ppm in the case of samples from areas of molybdenum excess.

Molybdenum reacts with thiocyanate in acid solution and in the presence of a reducing agent such as stannous chloride to give an amber colored solution. The colored compound of quinquevalent molybdenum with thiocyanate can be extracted from the aqueous phase with a

[4] Caution: Note comment on page 30 concerning evaporation of perchloric acid in hoods.

variety of organic solvents. Because most of the useful organic solvents, ether, isoamyl alcohol, etc., are lighter than water and are therefore difficult to separate cleanly from the aqueous phase, Johnson and Arkley (1954) recommended the use of a mixed solvent for extraction. The mixed solvent, an equal volume mixture of isoamyl alcohol and carbon tetrachloride, has a density greater than that of water and can be sharply separated from the aqueous phase using standard separatory funnel technique. This method is recommended for determinations of the highest order of sensitivity and accuracy. With careful attention to detail as little as 0.05 micrograms of molybdenum may be determined with 10 per cent accuracy. This amount corresponds to 0.01 ppm molybdenum when a five-gram sample is used. For many purposes, this order of sensitivity is unnecessary and an alternate procedure is given for use with samples where larger amounts of molybdenum are expected. For example in the determination of likelihood of field responses to molybdenum fertilization, i.e., in the range of 0.1 to 10 ppm, the original method is modified to use larger volumes of extractant and large (3 ml) spectrophotometer vessels.

Equipment

Wet digestion apparatus. See page 30 for details.

Separatory funnels. Borosilicate glass, Squibb type, 60-ml with tips cut off closely to stopcock. Permanently mark the separatory funnels to contain 45 ml of solution to facilitate use of nearly constant solution volumes. Separatory funnel shaker (Fig. 4) is useful but not required.

Burets. Fifty and 10 ml for dispensing reagents and solvent.

Spectrophotometer. Beckman Model B spectrophotometer, with $3 \times 10 \times 25$ mm. microcells.

Reagents

Water, redistilled from an all-borosilicate glass still, is used throughout the procedure.

Carbon tetrachloride-isoamyl alcohol. Mix equal volumes of A.C.S. reagent grade carbon tetrachloride and A.C.S. reagent grade isoamyl alcohol. The specific gravity of this solvent mixture is approximately 1.19.

Hydrochloric acid, approximately 6.5 N, containing ferric iron. Add 0.5 gram of ferric chloride hexahydrate to 560 ml of concentrated hydrochloric acid; dilute to 1 liter.

Sodium thiocyanate, 30 per cent, weight per volume. Dissolve 30 grams of sodium thiocyanate in water and make to 100 ml volume.

Stannous chloride, 40 per cent weight per volume. Suspend 40 grams of stannous chloride dihydrate ($SnCl_2$-$2H_2O$) in 20 ml of 6.5 N hydrochloric acid. Add redistilled water to dissolve and make to 100 ml volume. Filter if turbid.

Standard molybdenum solution. Dissolve 75 mg of pure molybdenum trioxide in a slight excess of sodium hydroxide, and dilute to slightly less than 500 ml. Make slightly acidic with hydrochloric acid and make to 500 ml with water. The above solution contains 100 micrograms of molydenum per milliliter. More dilute solutions may be prepared by appropriate dilution with redistilled water. Sodium molybdate of established composition has also been used.

Procedure

The following procedure can be used for small samples.

Ashing of plant material. Plant material may be ashed either by the dry method or the wet ashing as described in the general section on ashing procedures on page 32. Dry ashing may be done in porcelain dishes in a muffle furnace at 650°C. The dry material should not be moistened with any solution such

as alcoholic sulfuric acid or magnesium nitrate solution because dissolved molybdenum may fuse to the dishes at ashing temperature. The ash is taken up in 10 ml of redistilled water and 10 ml of 6.5 N hydrochloric acid-ferric iron solution and after gentle warming to ensure complete solution is transferred to the separatory funnel.

The stopcocks and stoppers of the separatory funnels should be only lightly lubricated occasionally with silicone stopcock grease. With properly fitting stopcocks, it is best not to lubricate at all. The use of Teflon stopcocks eliminates all lubrication need. The funnels should be washed with ethyl alcohol and redistilled water before each determination.

To the sample or standard in the funnel add 10 ml of the 6.5 N hydrochloric acid-iron solution if not already added in taking up the dry ash, and redistilled water to the 45 ml mark. If wet digestion is used, take into account the acidity introduced from the digest and reduce the amount of hydrochloric acid. The final acidity of the solution in the funnel may be varied between 0.7 and 1.8 N without affecting the color intensity.

Isoamyl alcohol is slightly soluble in the aqueous solution. The solubility varies with the salt content of the solution and, therefore, will be different for different sample sizes. In order to avoid this source of error, the aqueous phase must be saturated with isoamyl alcohol *before* adding the thiocyanate and stannous chloride solution. No molybdenum is lost when the excess of the saturating solution (isoamyl alcohol—carbon tetrachloride mixture) is discarded.

Saturate the aqueous phase by shaking vigorously with 3.0 ml of the isoamyl alcohol—carbon tetrachloride extractant. Allow 10 to 15 minutes for the phases to separate and quantitatively discard the extractant.

Add 1.0 ml of the 30 per cent sodium thiocyanate, mix and add 1.0 ml of 40 per cent stannous chloride solution and mix again. Add exactly 1.0 ml of the isoamyl alcohol-carbon tetrachloride extractant. Shake vigorously for 2 minutes, releasing pressure as necessary. Upon completion of the shaking period, wash out the tip of the funnel with redistilled water and dry with vacuum through a fine-tipped tube. Open the stopcock of the inverted funnel and dry the stopcock bore with the vaccum. Shake the funnel quickly and allow the phases to separate for about 15 minutes. Check the exit tip of the funnel again to remove traces of moisture. The above precautions are necessary to ensure that the colored extract be free of turbidity caused by traces of water. Discard 1 or 2 drops of the extractant and transfer the remainder directly to the spectrophotometer cell. Measure the absorbance of the solution at 470 mμ, using distilled water as reference. If the extract is too highly colored to permit accurate measurement of the absorbance, it may be diluted with the isoamyl alcohol-carbon tetrachloride extractant which has been treated by shaking with a solution containing the same concentrations of hydrochloric acid, sodium thiocyanate, and stannous chloride as was used in the determination.

Modified procedure, for use with samples high in molybdenum or when larger volumes of extract are required for spectrophotometer measurement.

In cases where extreme sensitivity is not required, it is more convenient to use larger volumes of extractant in the final extraction step. The use of two or more ml of extractant in the final step instead of one milliliter simplifies the procedure, making it easier to transfer the colored phase to spectrophotometer cuvettes. All other steps of the procedure are the same for the sensitive method. With some types of spectrophotometers or colorimeters, larger volumes of the carbon tetrachloride-isoamyl alcohol phase may be required to adequately fill the cuvettes. In this case it will be neces-

sary to employ a larger sample in order to maintain necessary sensitivity. For example, if 5 ml of extractant is required the minimum amount of molybdenum that can be detected with accuracy will be about 0.5 micrograms. Standards must be taken through the procedure using the same volume of extractant as used for samples. It is not permissible to extrapolate spectrophotometer readings from different extract volumes. Some crops show responses to molybdenum fertilization only when the molybdenum concentration in the tissues is lower than 0.1 ppm. Therefore, it would be necessary to use a 5 gram sample if the extractant volume were 5 ml in order to detect concentrations in the order of this value. It may be noted that alfalfa shows a response to molybdenum fertilization when the molybdenum concentration in leaf tissue is below 0.5 ppm.

Determination of Zinc by the Zincon Method

Amounts of zinc in plants range from 5 to 100 ppm in dry matter.

Until recently, zinc has been determined in plant material by measuring the color formed by zinc with dithizone. Dithizone also reacts with many heavy metals present in plant ash to form colored compounds which are extractable with carbon tetrachloride and elaborate precautions are necessary to prevent interference with the accurate measurement of the color due to zinc.

Recently Jackson and Brown (1956) have applied the method of Rush and Yoe (1954) to the determination of zinc in plant materials. This method uses a new reagent (Zincon) which forms with zinc a colored compound of such intensity that it is not necessary to extract with an organic solvent. Many ions in plant ash interfere in the color formation so it is necessary to separate the zinc from other ions. This is accomplished

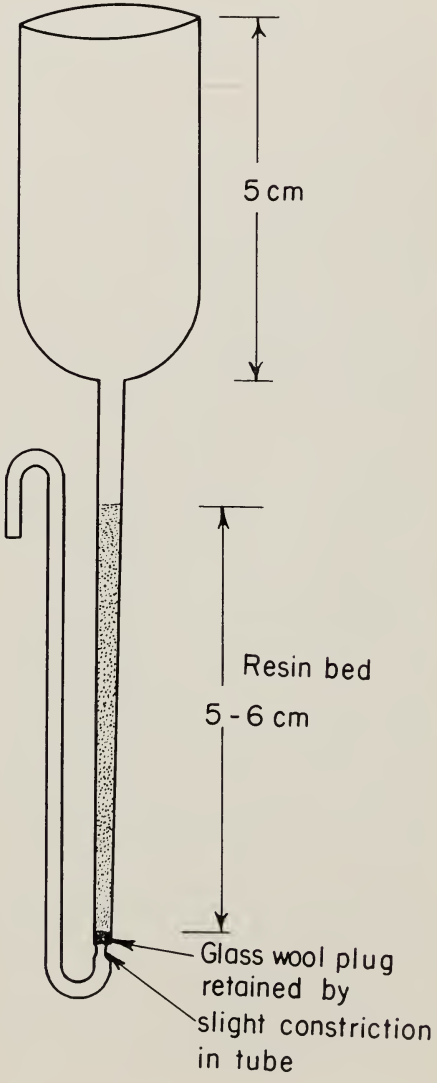


Fig. 5. Resin Column Funnel. The column is constructed of Pyrex or Kimax glass. Dimensions are not critical except that the top of the exit tube should be slightly higher than the top of the resin bed to prevent the liquid level dropping below the resin level.

If the liquid level is allowed to drop below the top of the resin bed air bubbles are trapped in the resin and the column does not function properly. Flow rate is controlled by adjusting the height of liquid in the funnel and should be about 1 ml. per minute. Faster rates may be tolerated in the adsorption step since zinc seems to be adsorbed rapidly but the elution step must not be too fast or the zinc will not be completely eluted.

in an ingenious way with an anion exchange resin column. The solution of plant ash in 2 N hydrochloric acid is passed through the column. Zinc forms a complex anion, probably $ZnCl_4^=$, which is quantitatively adsorbed by the resin. All interfering ions pass through the column. The $ZnCl_4^=$ is then quantitatively removed from the column by washing with 0.005 N hydrochloric acid or potassium chloride or, as modified in the author's laboratories, with 0.1 N sodium nitrate.

Addition of a buffer solution and the reagent, "Zincon," results in the formation of a blue color which may be measured in a colorimeter or spectrophotometer.

Equipment

Borosilicate 60° funnels, 50 mm top diameter, with stems cut to 3″.

Resin column funnels, according to design in sketch, Fig. 5.

Funnel supports.

Beakers, 20 or 30 ml.

Volumetric flasks, borosilicate, 50 ml volume.

Reagents

Dowex I.—Resin. Dowex I, ×-8, 500–100 mesh is suitable after treatment to remove traces of impurities.

Zincon Reagent.

Buffer Solution. Dissolve 31 grams boric acid and 37 grams of potassium chloride in 800 ml redistilled water. Adjust the pH of this solution to 9.2 with 6 N sodium hydroxide, approximately 50 ml are required. If the pH is too high, readjust with N hydrochloric acid. Make the buffer solution to a final volume of 1 liter.

Hydrochloric acid solutions. Assume concentrated hydrochloric acid to be 12 N.

Hydrochloric acid, 6 N. Dilute concentrated hydrochloric acid with an equal volume of redistilled water.

Hydrochloric acid, 2 N. Dilute 167 ml of concentrated hydrochloric acid to 1 liter with redistilled water.

Potassium chloride, 1 N. Dissolve 74.5 grams potassium chloride in redistilled water and make to 1 liter volume.

Sodium Nitrate, 0.1 N. Dissolve 8.5 grams sodium nitrate ($NaNO_3$) in redistilled water and make to 1 liter.

Buffer-Zincon Solution. Dissolve 0.125 gram of the Zincon powder in 250 ml of the pH 9.2 borate-potassium chloride buffer. This solution is stable for four or five days and should be prepared in amounts only sufficient for anticipated needs for this time.

Zinc Standard for Stock Solution, 1 ml contains 1 mg zinc. Dissolve 440 mg zinc sulfate heptahydrate ($ZnSO_4 7H_2O$) in 2 N hydrochloric acid and make to 100 ml volume in volumetric flask with the 2 N acid.

Working solution A, 1 ml contains 100 micrograms of zinc. Transfer 10 ml of the stock solution to a 100 ml volumetric flask and make to the mark with 2 N hydrochloric acid.

Dilute working solution B, 1 ml contains 5 micrograms of zinc. Transfer 5 ml of working solution A to a 100 ml volumetric flask and make to the mark with 2 N hydrochloric acid.

General caution. All glassware must be of Pyrex or Kimax and thoroughly washed with acid and redistilled water. Avoid rubber stoppers, stopper ties, etc., as rubber is likely to introduce zinc contamination.

Preparation of resin columns. Place a small plug of Pyrex glass wool at the base of the tube as illustrated in Fig. 5. Prepare a slurry of the Dowex I resin in water and pour into the tube until the resin height is approximately 5± 1 cm. Leach the column with 20–25 ml of concentrated hydrochloric acid, followed by like volumes of 6 N and 2 N hydrochloric acid. Finally pass 50 ml of the redistilled water through the column. Discard all the above washings.

Normally a column operates satisfactorily for about 20 determinations after which the resin should be replaced with new resin. Columns are normally prepared in batteries of six or twelve.

Preparation of Standard Curves. Wash each column with 5 ml of 2 N hydrochloric acid, discarding the washings. Transfer to the columns standard amounts of the dilute working solution B, as for example 1, 2, 5 and 10 ml. Also run blanks using similar volumes of 2 N hydrochloric acid. After all the 2 N acid has run through, pass 20 ml of the N potassium chloride solution through the columns and discard the effluent. Place a 50 ml volumetric flask under the column and pass two 20 ml portions of the 0.1 N sodium nitrate through the column to elute the zinc.

Regenerate the columns for the next run by passing 35 ml of the 2 N hydrochloric acid, discarding the effluent.

Development of the Zinc-Zincon color. Add to the volumetric flask, containing the 40 ml of 0.1 N sodium nitrate eluate, 5 ml of the buffer-Zincon solution, make to volume with redistilled water and mix thoroughly. Transfer the colored solution to a spectrophotometer or colorimeter cell and read the absorbance at 620 mμ. Plots of absorbance (corrected for the absorbance of the blank) against micrograms of zinc taken through the procedure should give a straight line. The color is stable for at least 12 hours after development. Rapid fading of the color is evidence that the buffer-Zincon solution is too old. The determinations should be repeated with freshly prepared buffer-Zincon solution. The blanks will give small but constant readings on the spectrophotometer because the Zincon reagent itself absorbs some light at 620 mμ.

Determination of zinc in plant samples. The method as described by Jackson and Brown (1956) specifies dry ashing of the plant material. As noted previously, dry ashing of plant material invariably leaves a residue of silica which adsorbs traces of micronutrients. This is the case of dry ashing of samples for zinc. Studies in the authors' laboratories show that losses by adsorption on silica may be as high as 10 to 15 per cent unless the silica is removed by treatment with hydrofluoric acid. For many practical purposes, especially where samples of the same type of material are to be compared, this loss may be sufficiently constant to allow useful comparisons. For this reason, the method of Jackson and Brown (1956) will be given as originally presented. In addition, special directions will be given for determination of zinc by the Zincon method on aliquots from wet digestions.

Transfer 1.00 gram of the ground or crushed sample to a 20 ml beaker and ash at 500°C for 2 hours. Moisten the cool ash with a few drops of water and add 5 ml of the 2 N hydrochloric acid, warm gently for a few minutes to facilitate solution of the ash.

Filter the ash solution through 9 cm No. 30 filter paper which has been thoroughly washed with hot 2 N hydrochloric acid. Catch the filtrate directly on the freshly regenerated resin column. Wash the beaker and filter paper with cold 2 N hydrochloric acid also directly into the resin column. Total volume of ash solution and washing need not exceed 30 to 40 ml if care is taken to wash with several small portions of 2 N hydrochloric acid. Continue with the determination as described for standards.

Special procedure for determination of zinc after sample has been ashed by nitric-perchloric acid wet digestion.

Perchloric acid interferes in the adsorption of zinc by the anion exchange resin and must be removed prior to determination of zinc. (Perchlorate does not interfere in the development of the color but only in the adsorption of zinc on the resin.) Transfer an aliquot of the wet digest (done according to directions, page 32) to a 50 ml beaker, add one

drop of concentrated sulfuric acid, evaporate to dryness and fume off perchloric acid by heating on an electric hot plate or over a Meeker burner. Make certain that all fumes that have condensed on the sides of the beaker are removed. This fuming has converted ortho-phosphate to pyro-phosphate which forms an insoluble compound with zinc. Therefore, the pyrophosphate must be hydrolyzed to orthophosphate. This is done by adding 10 ml of the 2 N hydrochloric acid to the beaker, covering with a watch glass and heating on the steambath for one half hour. Transfer this solution to the resin column and proceed as directed with the adsorption on the resin column, elution, and color development.

Determination of Zinc by the Dithizone Method

Extreme ranges of zinc concentration in plant materials are about 5 to 100 ppm. Most frequent values are 10 to 40 ppm.

The dithizone method (Cowling and Miller, 1941) is a sensitive and accurate method for zinc provided careful attention is paid to detail.

Equipment

All glassware must be of borosilicate formulations (Pyrex or Kimax) and thoroughly washed to remove traces of adsorbed heavy metals. Wash with molar potassium or ammonium ethylenediaminetetraacetate solution (pH about 8–8.5), rinse with glass distilled water and wash with warm 3 N hydrochloric acid and thoroughly rinse with glass distilled water. The use of chromic acid cleaning solution is not necessary unless the glassware is contaminated with greasy residues that cannot be removed by other treatments. If chromic acid cleaning solution is used, extreme care must be taken to remove all traces of chromate because traces of chromate oxidize dithizone.

Separatory funnels, 120 ml, pear shaped or Squibb type are satisfactory. If the stopcocks fit properly they may be used without lubrication. If necessary to lubricate, use a minimum of grease that has been tested for freedom from heavy metals. Separatory funnel shaker (Fig. 4) is useful but not required.

Kjeldahl flasks, 30 ml or larger if necessary. Thirty ml flasks are large enough if 1 gram samples are wet ashed and care is taken to avoid foaming in the initial stages of the wet digestion.

Spectrophotometer or photoelectric colorimeter. If a filter type colorimeter is used, the Sextant green (Corning No. 401) filter transmits the proper wave length of 535 mμ.

Reagents

Water used in preparation of reagents and ash solutions must be free of zinc and other heavy metals. Usually it is necessary to distill the laboratory supply of distilled water from an all-glass still. Reagents and water must be stored in borosilicate glass bottles.

Acids and other reagents must be of highest analytical grade. Reagents labeled "A.C.S." or "analytical grade" are usually quite free of heavy metals. However, blanks should be run on all reagents. If blanks appear to be high, tests of individual reagents should be made and appropriate purification measures taken.

Ammonium Hydroxide. Distill ammonium hydroxide from an all-glass still and dilute to 1 molar.

Hydrochloric acid. Dilute concentrated hydrochloric acid with an equal volume of distilled water and distill from an all-glass still. Discard the first portion and collect the constant boiling portion which will be 6 normal. Dilute to 1 N with redistilled water.

Hydrochloric acid, 0.002 N. Dilute 100 ml of N hydrochloric acid to 5 liters with redistilled water.

Dithizone solution in carbon tetrachloride. Dissolve 0.2 grams of dithizone in 500 ml of carbon tetrachloride, filter if necessary to remove insoluble matter. Place the solution in a large separatory funnel and add 2 liters of 0.02 molar ammonium hydroxide. Shake the funnel vigorously to extract the ammonium dithizonate into the aqueous phase, discard the carbon tetrachloride and extract the aqueous phase with 100 ml portions of carbon tetrachloride until the carbon tetrachloride phase is pure green. The extractions serve two purposes: first to remove any oxidized dithizone and secondly to remove any heavy metal dithizonates that may have been present. All carbon tetrachloride phases to this point are discarded. Add to the aqueous phase 500 ml of carbon tetrachloride and 45 ml of molar hydrochloric acid and shake the funnel to extract the acid form of dithizone into the carbon tetrachloride. Transfer the carbon tetrachloride phase to a clean 2 liter volumetric flask and make to volume with carbon tetrachloride. Transfer this solution to a bottle and store in the dark in a cool place.

Ammonium Citrate Solution, 0.5 molar. Dissolve 226 grams of ammonium citrate, $(NH_4)_2HC_6H_5O_7$; or 210 grams of citric acid monohydrate $H_3C_6H_5O_7$-H_2O, in about 1,500 ml of distilled water, add concentrated ammonium hydroxide until the pH is about 8.5–8.7. (Note that it is not necessary to use glass distilled water or ammonium hydroxide in the preparation of this solution because the heavy metal contamination that is always present in the citrate is later removed by dithizone extraction.) Add a small excess of dithizone, as shown by the yellow-orange color remaining in the aqueous phase after shaking with carbon tetarachloride, and extract with successive 100 ml portions of carbon tetrachloride until a pure green color remains in the carbon tetrachloride phase and the aqueous phase still has a pale yellow color. Dilute to 2,000 ml and store the heavy metal-free aqueous phase in a borosilicate bottle.

Carbamate reagent. Prepare fresh daily before use. Dissolve 0.25 grams of sodium diethyl-dithiocarbamate in 100 ml of glass distilled water.

Solution A. Dilute 1 liter of the 0.5 M ammonium citrate and 140 ml of M ammonium hydroxide to 4 liters.

Solution B. Dilute 1 liter of the 0.5 M ammonium citrate and 300 ml of M ammonium hydroxide to 4.5 liters.

Solution C. Immediately before use, mix 1 volume of freshly prepared carbamate with 9 volumes of solution B. Fifty ml of solution C are required for each sample, standard, and blank, therefore, if 5 standards, 2 blanks, and 20 samples are to be done on a given day, one would mix 140 ml of 0.25 per cent carbamate solution and 1,260 ml of solution B thus providing 50 ml for each determination with a small amount left over.

It is noted that large volumes of solutions A and B and 0.02 M hydrochloric acid are prepared. This procedure provides for uniformity of reagents used in the determination for a considerable period of time. Once confidence in the technique has been developed, it is possible to dispense with running blanks or standards with each run of samples. However, when new reagents are prepared, blanks and standards should be run to reestablish the calibration curve. It should be borne in mind that solutions will dissolve some zinc on standing, even in borosilicate glass, so that the blank value of the reagents will increase slowly with time. Prepare solutions in amounts large enough only to meet anticipated needs for two or three months. Never store in soft glass containers.

Zinc standard, Stock solution. 1 ml contains 1 mg zinc. Dissolve 440 mg zinc sulfate ($ZnSO_4 7H_2O$) in 2 N hydrochloric acid and make to 100 ml volume in volumetric flask with the 2 N hydrochloric acid.

Working solution I, 1 ml contains 100 micrograms of zinc. Transfer 10 ml of the stock solution to a 100 volumetric flask and make to the mark with 2 N hydrochloric acid.

Dilute working solution. II, 1 ml contains 5 micrograms of zinc. Transfer 5 ml of working solution I to 100 ml volumetric flask and make to the mark with 2 N hydrochloric acid.

Procedure

Transfer 1.00 gram of sample to a 30 ml micro-Kjeldahl flask, add one Hengar boiling chip, 10 ml concentrated nitric acid and 2 ml perchloric acid and allow to stand overnight or heat carefully at first to avoid foaming over. Complete the digestion as described on page 32. Fume off most of the perchloric acid, but do not take to complete dryness, leaving a moist residue of perchlorate salts. Add 10 ml glass distilled water and boil gently for 10 to 15 minutes. Dry ashing of plant material for zinc determination is not recommended unless platinum ashing dishes are used so that the silica residue can be removed with hydrofluoric acid.

Transfer the ash solution to a 100 ml borosilicate volumetric flask and make to volume with glass distilled water. Transfer a 50 ml aliquot of the ash solution to a 120 ml separatory funnel. The aliquot taken should be such that it contains from 5 to 30 micrograms of zinc. If the 50 ml aliquot contained too much zinc, repeat the determination with a smaller aliquot.

Add 1 drop of phenolphthalein and ammonium hydroxide until the solution is a faint pink. Add 40 ml of solution A and 10 ml of the dithizone reagent and shake vigorously for ½ minute. The aqueous phase should be orange-yellow in color. If not, add more dithizone solution in 1 ml increments until the aqueous phase remains colored. Shake the funnel vigorously for an additional 2 minutes. (See Figure 4 for details of construction of simple separatory funnel shaker.) Allow the phases to separate, shaking down the drop from the top of the aqueous phase, and run the carbon tetrachloride phase off into another separatory funnel. Rinse the aqueous phase in the funnel with a few milliliters of fresh carbon tetrachloride, adding these rinsings to the second funnel. Do not allow any aqueous phase to pass into the second funnel with the carbon tetrachloride solution because the aqueous phase is strongly buffered at an alkaline pH and will prevent proper pH control in the separation of copper and zinc when the dithizone extract is shaken with hydrochloric acid.

Repeat the extraction of the aqueous phase with successive 5 ml portions of carbon tetrachloride until the carbon tetrachloride phase has only a pure green color and the aqueous phase is still orange-yellow in color. If necessary, add more dithizone solution to ensure excess of dithizone in the aqueous phase. Collect all carbon tetrachloride phases in the second separatory funnel.

To the second separatory funnel containing the carbon tetrachloride solution of zinc and copper dithizonates, pipet 50 ml of the 0.02 N hydrochloric acid and shake the funnel vigorously for at least 2 minutes. Allow the phases to separate, shake down the drop of carbon tetrachloride from the top of the aqueous phase and draw off and discard the carbon tetrachloride containing free dithizone and copper dithizonate. Rinse the aqueous phase with two or three portions of carbon tetrachloride. Again the separations must be sharp because the aqueous phase contains all the zinc and because all the carbon tetrachloride must be removed before adding a measured volume of dithizone solution in the next step. Likewise, the drop of carbon tetrachloride that always remains on top of the aqueous phase must be shaken down and discarded. Pipet 50 ml of solution C (carbamate in citrate buffer) into the separatory funnel containing the zinc in

0.02 N hydrochloric acid. The volumes of 0.02 N hydrochloric acid and solution C must be accurately measured in order that the final pH be held constant at 8.5–9.0 in all determinations of samples and standards. Add exactly 10 ml of the dithizone solution and shake vigorously for two minutes. Allow the phases to separate. Draw off and discard a few drops of the carbon tetrachloride phase (to clear the stopcock of aqueous phase). Draw off the remainder of the carbon tetrachloride phase into a clean test tube. Transfer a 5 ml aliquot of the carbon tetrachloride solution of zinc dithizonate to a 25 ml volumetric flask, make to the mark with carbon tetrachloride, mix and transfer the solution to the spectrophotometer cuvette. Read absorbance, using a wave length of 535 mμ; with a filter photometer use the sextant green (Corning No. 401) filter. The solutions should be protected from strong light prior to measurement. The carbamate is used in the final extraction to prevent the formation of dithizonates of heavy metal other than zinc. Because carbamate does slightly reduce the zinc dithizonate extraction it must be used in standard and blank determinations as well as in the samples. Standards and blanks are run in the same manner as described above.

In order that the information in our publications may be more intelligible, it is sometimes necessary to use trade names of products and equipment rather than complicated descriptive or chemical identifications. In so doing, it is unavoidable in some cases that similar products which are on the market under other trade names may not be cited. No endorsement of named products is intended nor is criticism implied of similar products which are not mentioned.

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